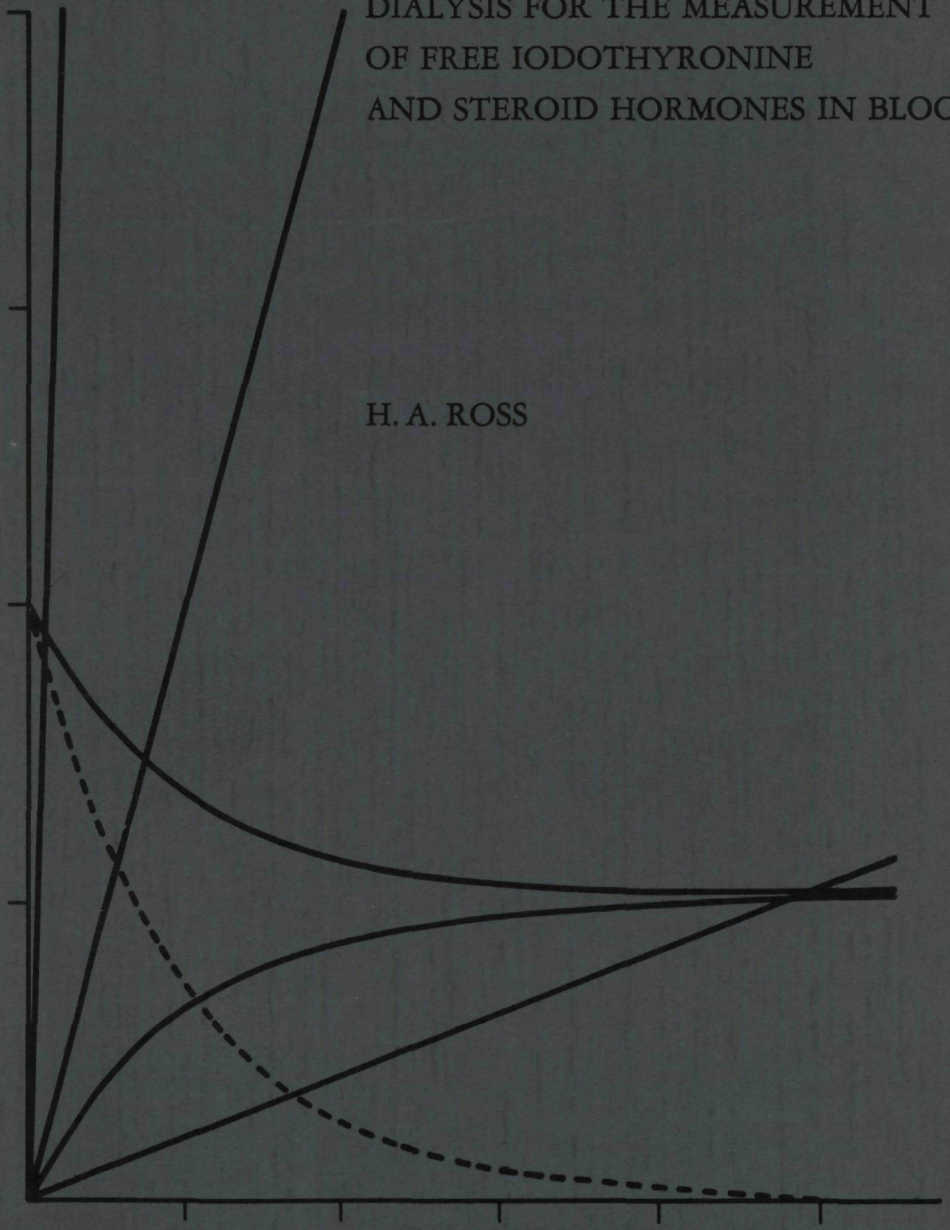
SYMMETRIC AND EQUILIBRIUM DIALYSIS FOR THE MEASUREMENT OF FREE IODOTHYRONINE AND STEROID HORMONES IN BLOOD

H. A. ROSS



SYMMETRIC AND EQUILIBRIUM DIALYSIS FOR THE MEASUREMENT OF FREE IODOTHYRONINE AND STEROID HORMONES IN BLOOD

PROMOTORES

PROF DR TH J BENRAAD

PROF DR P W C KLOPPENBORG

SYMMETRIC AND EQUILIBRIUM DIALYSIS FOR THE MEASUREMENT OF FREE IODOTHYRONINE AND STEROID HORMONES IN BLOOD

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS PROF DR P G A B WIJDEVELD,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG 17 APRIL 1980,
DES NAMIDDAGS TE 2 00 UUR PRECIES

DOOR

HENRY ALEXANDER ROSS

GEBOREN TE AMSTERDAM

DRUKKERIJ-UITGEVERIJ
BRAKKENSTEIN
NIJMEGEN

Het in dit proefschrift beschreven onderzoek werd verricht in de afdeling Experimentele en Chemische Endocrinologie (Prof. dr Th J. Benraad) en in het laboratorium voor Endocrinologie van de Kliniek voor Inwendige Ziekten van het St Radboudziekenhuis te Nijmegen.

Lectori Salutem!

CONTENTS

(continued overleaf)

Introduction.

The concentrations of the free, non-protein-bound iodothyronine and steroid hormones in blood are considered to reflect their biological action more closely than total levels, because a) the free form of a hormone is able to diffuse into cells to exert its action, b) it is an important determinant of hormone turnover and c) alterations in binding protein concentrations which indirectly influence total hormone levels, generally do not or to a much lesser extent, affect free concentrations.

Until now no methods are known for the measurement of free hormones in blood without prior separation of free and protein-bound hormone. After separation either the free hormone is measured directly, e.g. by radioimmunoassay, or the proportion of hormone that is free i.e. the free hormone fraction, is measured. This is done by adding a tracer amount of the hormone in radio-active labelled form before free and protein-bound hormone are separated. As it is assumed that radioactive and non-radioactive hormone are equally partitioned over free and protein-bound moieties, the distribution of radioactivity after separation will yield the free hormone fraction. This free hormone fraction must be multiplied by the total hormone concentration in order to obtain the true free hormone concentration. The crucial step for free hormone determinations is the technique for separation of free and bound.

There are many separation methods which are quite satisfactory for radioimmunoassay or measurement of concentration of binding sites by ligand-binding e.g. in receptor assays. However, most of these methods are not accurate enough for free hormone determinations.

The well-known charcoal separation technique for instance, may lead either to underestimation of the free hormone as a result of incomplete adsorption of free, or to an overestimate if the hormone-protein complex is also adsorbed to some extent.

An overestimation may also be the result of continuous displacement of the hormone-protein equilibrium leading eventually to

complete "stripping" of the proteins. Errors of this type are not particularly troublesome in radioimmunoassays as long as they do not exceed say 10% and if they are constant and equal for standards and samples. In contrast, measurement of a free hormone fraction of which the true value is 0 01 is already impossible when the applied technique separates bound with an efficiency of 99% (e.g. a device for concentrating protein solutions by rapid ultrafiltration). As 1% of the bound is estimated as free, the observed value will be twice as high as the true one. Equilibrium dialysis, ultrafiltration and gel equilibration are, until now, considered as the only separation techniques that may yield accurate results when applied for free hormone assay.

Basically, dialysis is a technique for the separation of large and small molecules by means of a semipermeable membrane. The principle can be applied in several forms, depending on the problem at hand. Of these forms almost exclusively the equilibrium dialysis method has been used for free hormone assay. The main application for equilibrium dialysis is the study of ligand-protein interaction and as such it is widely used for assesment of the hormone-binding properties of serum proteins (Westphal 1971). Equilibrium dialysis makes use of two compartments of fixed size which are separated by a semipermeable membrane. The system may consist of dialysis tubing containing one solution immersed in the other, or it may be a specially designed dialysis cell having precise and reproducible properties. One compartment contains a solution of both ligand and binding proteins(e.g. serum, including free and bound hormone and hormone-binding proteins) and is referred to as the dialysand. The solution in the other compartment (dialysate) does not contain any binding protein but is accessible to the non-protein-bound hormone which can freely diffuse through the membrane whereas the larger protein molecules are excluded. Final equilibrium is reached when the concentration of free hormone is the same in both compartments so that no diffusion takes place any more. As a first approximation the hormone concentration in the dialysate is considered to correspond to the free hormone con-

centration in serum. Likewise the ratio of labelled hormone concentration in the dialysate to that in the dialysand is assumed to reflect the original free hormone fraction in serum. Although measurements take place at equilibrium, the dialysed sample has been subjected to change since some hormone has diffused out of the serum compartment, which must have an effect on the hormone-protein equilibria involved and thereby on the free hormone concentration. This influence of mere application of the technique itself on the free hormone concentration, which can be shown to be equivalent to a dilution effect, has been overlooked by a majority of authors. Also in instances where the effect of dilution on the free concentration of a certain hormone was studied (Oppenheimer et al, 1963, 1964; Vermeulen et al, 1971), no explicit analysis of the effect of dialysis itself was made. In chapter I such an analysis is made by means of a mathematical model for hormone interaction with multiple binding sites. Also other problems involved in equilibrium dialysis, such as the migration of solvent towards the dialysand because of osmotic pressure (Schlussler et al, 1967; Lutz et al, 1977), or the effect of dilution of the sample before dialysis to prevent such fluid shift (Oppenheimer et al, 1964; Vermeulen et al, 1971; Kley et al, 1977; Moll, 1978) could be tackled with result by means of this model. Simultaneously the intrinsic limitations of the technique to provide an accurate estimate of the free hormone concentration in the original, undiluted serum sample, as a consequence of the fact that dialysis always involves some dilution and concomitant shift of hormone, were better defined. These limitations were one reason to look for an alternative method that would not affect the sample to the same extent. The other reason to look for an alternative method was the extreme susceptibility of some free hormone assays, particularly those of thyroxine (T4) and 3. 5,3' triiodothyronine (T3), to radiochemical contamination of the used tracer preparation, which necessitates tracer purification both before and after dialysis (Oppenheimer et al, 1963; Sterling et al, 1966; Herrmann et al, 1971; Cavalieri et al, 1975). Direct assays of these hormones in equilibrium dialysates, which do not suffer from this type of interference have been described (Ekins et al, 1975; Yeo et al, 1977;Kristensen et al, 1977) but the highly sensitive radioimmunoassays required are not

readily available. The disturbance caused by tracer contamination depends for the larger part on the proportion of radioactive impurity to the radioactivity of the intact tracer hormone in the dialysate. In addition to purification steps to reduce the contamination one can also increase the contribution of intact tracer to the dialysate radioactivity by diluting the sample (Oppenheimer et al. 1963; Herrmann et al, 1971) which results in a higher free hormone fraction and thereby in a higher free tracer hormone fraction. The contaminating material is expected not to be bound to serum proteins or only slightly, so that dilution of the binding proteins does not affect the concentration of the impurities, which consequently are almost or completely evenly distributed throughout both dialysis compartments. Still, this method remains cumbersome and susceptible to interference. Therefore, a new method must be one in which the tracer distribution over the two dialysis compartments, which always is some measure of the free hormone fraction, at the moment of its measurement is hardly affected by contaminants.
The method that has been developed to be liberated from these problems is based on assessment of the dialysis rate in a dialysis system that contains identical solutions on both sides of the membrane and therefore was denoted as "symmetric dialysis". The principle of the method has been described (Ross, 1978) as well as some applications (Ross, 1979). It consists of dialysis of diluted or undiluted serum containing a tracer amount of radioactive labelled iodothyronine or steroid hormone against an identical sample without added tracer. This will ultimately result in equal distribution of radioactivity over the two dialysis compartments. The rate at which this equilibrium state is approached depends not only on the permeability of the dialysis membrane, its area and sample volume, but also on the fraction of added tracer that is not bound to serum proteins. As labelled and unlabelled hormones are equally partitioned over free and protein-bound forms, the dialysis rate of tracer hormone depends on the free hormone fraction. This may be understood from the fact that half of the amount of tracer present must migrate to the other compartment, but it can diffuse through the membrane only when it is in the free form. Consequently, when only a small proportion of the hormone is free, it will take longer before final equilibrium is reached than with

higher free hormone fractions. The dialysis rate is conveniently expressed by the (radioactive) isotope distribution as a function of time. A mathematical expression, describing the relationship between tracer distribution, dialysis time, membrane permeability, free hormone fraction and other constants of the system can be readily derived. This is done in chapter II. Methodologically, this dialysis rate method compares favourably with conventional equilibrium dialysis in the following respects: Except for the tracer, the solutions on both sides of the membrane are identical, which means that a) the composition of these solutions is constant during dialysis and b) no osmotic pressure differences and Donnan effects can occur, which in equilibrium dialysis may lead to dilution of dialysand and unequal distribution of free hormone over dialysand and dialysate (Westphal, 1971). Furthermore, c) the presence of binding proteins on both sides of the membrane completely prevents adsorption of ligand to membrane or cell walls, and d) the method is intrinsically less susceptible to interference by radioactive contaminants of the tracer because a much larger proportion of (intact) tracer migrates to the other compartment than with the equilibrium method. So,tracer impurities affect the observed tracer distribution to a much lesser degree. This is of particular importance for free T3 and even more for free T4. The basic principle of symmetric dialysis depends on the assumption that the rate of tracer transfer is determined entirely by diffusion through the membrane. Experiments showed however that under certain circumstances the dialysis rate was affected by factors influencing tracer transport in the solution adjacent to the membrane. Therefore the derivation of the basic principle of the method in chapter II was extended to include the possibility that the rate of tracer transport is also influenced by processes other than membrane diffusion. Also the possibility that association or dissociation rates of the hormone-protein complexes influence the overall dialysis rate is fitted into the mathematical model of symmetric dialysis.

Besides a brief summary of methods used in the ensuing chapters, chapter III is meant to provide a practical guideline for performing the simpler free hormone assays by symmetric dialysis and by equilibrium dialysis according to the views developed in chap-

ters I and IV. The latter chapter starts with derivation of simple, straightforward procedures for evaluating free hormone concentrations and fractions from equilibrium dialysis data, based on the theory in chapter I. This is followed by a critical discussion of existing methods. Subsequently some crucial points of the developed concept are experimentally verified. In chapter V essential points of the theory of symmetric dialysis from chapter II are experimentally tested and the usefulness of the method is demonstrated by some applications.

This thesis is meant as a study of methods in the first place. New insights in the role of free hormones and the significance of hormone binding to serum proteins cannot be directly derived from it. Still it is hoped that the reader, whose interests lay mainly in that field, will find enough material, in the form of the relations and processes in which the free hormone participates, to stimulate his imagination. It is also hoped that he will be intrigued and challenged by the problem of quantitation of something that hides itself from observation like the free hormone concentration.

Chapter I

FREE HORMONE ASSAY BY EQUILIBRIUM DIALYSIS: REVISION OF THEORETICAL FUNDAMENTALS.

I.1. Introduction.

Equilibrium dialysis, along with ultrafiltration, is considered to be the most reliable method for separating free and bound hormonal ligand (steroid or iodothyronine) in multiple binding site systems. Although it does influence the binding equilibria involved it does so in a controlled way: a new equilibrium results which is not identical, but closely related to the original state prior to dialysis.

The free hormone concentration in the undiluted, original serum sample is generally assumed to have the greater physiological significance, so the relation between what is measured at dialysis equilibrium and the true concentration of free hormone in the original sample is of great importance. When a sample is brought into contact with a certain volume of saline or buffer by means of a membrane which allows passage to solvent and small solute molecules, but not to (protein) macromolecules, one can think of the sample as being affected by two processes: first, a certain amount of hormone diffuses through the membrane to the other compartment (dialysate) resulting in a net decrease of the total hormone concentration in the serum compartment (dialysand) and second, the distribution volume for all small molecules present is enlarged, which might be conceived as a dilution.

After derivation of a mathematical model for hormone-protein interaction (I.2.), this chapter deals with the examination of the influence of changes in total hormone concentration (I.3.) and dilution (I.4.) on the concentration of free hormone.

The equilibrium dialysis process is described in terms of the mathematical model from which it is found that, with regard to the concentration and fraction of unbound hormone, a complete analogy exists with mere dilution (I.5.). Therefore it is concluded that equilibrium dialysis is most adequately described as a dilution

of the (e.g. serum) sample and that relationships which hold for dilution, also may be applied for equilibrium dialysis. Finally, in the appendix (I.A,B) the mathematical model is used to simulate the plasma multiple binding site system for some hormones in worked out examples.

I.2. A mathematical model for hormone-protein interaction.

The mathematical model for multiple hormone-protein equilibria that will be used to study the effects of separating free hormone from a sample by means of a semipermeable membrane may be viewed as a intermediate between a model which deals with one ligand order of binding sites (e.g. Mills, 1962) and the m ligands, n orders of binding sites model by Feldman (1972). Furthermore the model is extended in order to study the effects of dilution on the free hormone concentration. It is derived as follows:
Let hormone (H) and protein (P) react reversibly according to the law of mass action to form a complex (HP):

$$H + P \leftrightarrows HP \qquad (I\text{-}1)$$

The equilibrium (dissociation) constant of the reaction is given by:

$$\frac{[H][P]}{[HP]} = K \qquad (I\text{-}2)$$

(I-1) and (I-2) represent the interaction of a single species of (hormonal) ligand with a single order or class of binding sites. The law of mass action (I-2) will also hold when n orders of binding sites i (i = 1 .....n) are present. When it is assumed that all binding sites are independent, i.e. occupation of one site does not affect binding affinity of another site for the ligand, it is not essential to the model whether some orders of binding sites are located on the same protein or not.
The following symbols are introduced:

F = concentration of free, unbound hormonal ligand.

B_1 = " of hormone, bound to binding sites 1.

P_1 = " of binding sites 1 (="maximal binding capacity")

K_1 = dissociation constant of the complex-formation reaction between hormone and binding sites 1.

H = total hormone concentration.

f = F/H = free hormone fraction.

b = $1 - f$ = bound hormone fraction.

Using these symbols, (I-2) can be rewritten to apply to binding sites 1. Since $[H] = F$, $[HP] = B_1$, and $[P]$ represents the concentration of unoccupied sites so $[P] = P_1 - B_1$, one obtains:

$$\frac{F(P_1 - B_1)}{B_1} = K_1 \qquad \text{(I-3)}$$

Rearrangement of (I-3) gives an expression for the concentration of hormone bound to sites 1:

$$B_1 = \frac{P_1 F}{K_1 + F} \qquad \text{(I-4)}$$

Summation of all bound concentrations and the free hormone concentration gives for n orders of binding sites the total hormone concentration H:

$$H = \sum_{1=1}^{n} B_1 + F \qquad \text{(I-5)}$$

Combined with (I-4):

$$H = F \sum_{1=1}^{n} \frac{P_1}{K_1 + F} + F \qquad \text{(I-6)}$$

This general expression forms the basis of all further studies on factors that influence free hormone concentration. It is employed for studying the effect of changing the total hormone concentration (I.3.), it is adapted for the examination of the influence of dilution (I.4.), it offers the possibility to describe equilibrium in terms of binding parameters (I.5.) and finally it serves as the basic relationship from which improved calculation procedures for free hormone estimation from experimental data are derived (IV.2.).

I.3. Free hormone concentration dependence on total hormone.

The general expression (I-6) relates free to total hormone in a complex binding system. It offers the possibility of studying the change in free hormone concentration when total hormone in the system is changed. This expression is cumbersome because of the summation of terms (expressions for B_1) which are non-linear in F, so that F cannot be made explicit. However, limiting conditions can be found for which such non-linear terms reduce to linear ones. These limiting conditions will be used both in order to derive relations that are useful to describe effects of applying dialysis and as an aid to make the study of a complex hormone-binding system by means of simulation accessible to researchers with limited (electronic) memory space.

Expression (I-4) for the concentration of hormone bound to sites of order 1 reduces to a linear one for extremely low and extremely high values of F.
When the condition:

$$F \ll K_1 \qquad \text{(I-7')}$$

holds, eq. (I-4) reduces to

$$B_1 = F \frac{P_1}{K_1} \qquad \text{(I-7a)}$$

Interestingly, the concentration bound (B_1) has become directly proportional to the free hormone (F) and furthermore, it is easily seen that the ratio of occupied binding sites (B_1) to total sites available (P_1) which may be termed occupancy or degree of saturation, has become equal to F/K_1 which approaches zero since $F << K_1$. Should the limiting condition apply for all orders of binding sites $i = 1 \ldots\ldots n$, (I-6) would reduce to:

$$H = F \sum_{i=1}^{n} \frac{P_i}{K_i} + F \qquad \text{(I-7b)}$$

Under these circumstances, also free (F) and total hormone concentration (H) are directly proportional. It will be shown that this expression is adequate for description of the dependence of free on total aldosterone in serum for a large concentration range (appendix IA.1.1).
For the opposite condition:

$$F >> K_i \qquad \text{(I-8')}$$

(I-4) changes to:

$$B_i \approx P_i \qquad \text{(I-8a)}$$

The concentration of hormone bound to sites of order i now has become independent of F, simply because alle sites are occupied as $B_i/P_i = 1$. Should this apply for all sites, eq. (I-6) would change to

$$H = \sum_{i=1}^{n} P_i + F \qquad \text{(I-8b)}$$

showing that further increase of total hormone H only would result in a rise of F by means of a linear relationship.

For the application of simplifications of the expression for B_i

for values of F between the extremes that lead to (I-7b) and (I-8b), it will be convenient to define the limiting conditions more precisely by choosing a fixed criterion for the relative magnitudes of F and K_1. When a ratio of hundred is chosen, conditions (I-7') and (I-8') become

$$F < 0.01\ K_1 \qquad \text{(I-9a)}$$

and

$$F > 100\ K_1 \qquad \text{(I-9b)}$$

respectively. Substitution of $F = 0.01\ K_1$ in both (I-4) and (I-7a) shows that B_1 calculated from (I-7a) ($0.01\ P_1$) differs only by 1% from the B_1 value obtained with the general, non-linear expression (I-4) ($0.01\ P_1/1.01$). A difference of 1% is also obtained when $F = 100\ K_1$ is substitued in (I-8a) (expression does not contain F, so still $B_1 = P_1$) and in (I-4) ($B_1 = 100\ P_1/101$). It is readily seen that occupancies at these limiting conditions are 1% and 99% respectively.

When returning to the complicated expression (I-6) it may be assumed that for a certain value or range of values of F the condition $F > 100\ K_1$ holds for all orders of binding sites with i = 1 ... .. ℓ and the condition $F < 0.01\ K_1$ for all orders of binding sites with i = m .....n. Then the concentrations of bound hormone B_1 (i = 1 .....ℓ) may be written as a sum of terms of the form (I-8a) and B_1 (i = m .....n) as the sum of expressions according (I-7a).
The binding sites for which neither of the limiting conditions hold (i = ℓ+1 .....1) still require the non-linear notation for B_1. Summing the three types of expressions for B_1, eq. (I-6) becomes:

$$H = \sum_{i=1}^{\ell} P_i + F \sum_{i=\ell+1}^{m-1} \frac{P_i}{K_i + F} + F \sum_{i=m}^{n} \frac{P_i}{K_i} + F \qquad \text{(I-10)}$$

By this operation the number of non-linear terms is reduced from n to m - ℓ - 1. Of course the numbers of terms, that may be written in either linear form, depends on F. As shown before, the free hormone concentration may attain such values that all B_1 terms may be written in a linear form. One example of such a case is encountered for $F < 0.01\ K_1$, herewith assuming that the orders of binding sites are numbered in order of increasing K, so that $K_1 < K_{1+1}$ and K_1 is the lowest and K_n is the highest dissociation constant found in the binding system. So, (I-10) reduces to (I-7b) for all $F < 0.01\ K_1$. This limiting condition is frequently used in this study since it not only yields a simple relation between total and free hormone but also between dilution and the free hormone concentration. A practical instance of a binding system which is actually governed by (I-7b), is formed by aldosterone in normal serum (appendix IA.1.1).
In general, when departing from the situation where $F < 0.01\ K_1$, the free hormone concentration is gradually increased, first the order of binding sites with the highest affinity will become occupied to a significant degree (e.g. > 1%) so that for this order the non-linear expression must be used. One by one the other orders of binding sites will increasingly become occupied and the first order to become fully saturated will be again the one with the highest affinity (K_1). Thus, sequential saturation of the binding sites takes place with increasing F. This is illustrated in appendix IA.1.2 where the relation between total and free thyroxine (T4) in serum, in the presence of five different orders of binding sites, is worked out with help of eq. (I-10).

Another useful parameter which characterises the state of a hormone in a binding protein system is the free hormone fraction f (= F/h). This parameter is a dimensionless number between zero and unity which defines what proportion of the total hormone is present in the free, unbound form. It is of great practical importance since it provides an alternative way for assesment of the free hormone concentration when direct measurement is not feasible as a consequence of inadequate assay sensitivity. Instead the free hormone fraction is determined by measuring what proportion of added radio-

active tracer hormone is in the free form. Multiplication of the result with the total hormone concentration gives, according to the definition, the free hormone concentration F. In the worked out examples, which are in fact simulation experiments, the free hormone fraction is obtained from the opposite side since values of F are calculated first (appendix IA.1.1,2).
A general expression for f is readily derived from (I-6):

$$f = \frac{F}{H} = \frac{1}{\sum_{i=1}^{n} \frac{P_i}{K_i + F} + 1} \qquad \text{(I-11)}$$

Thus it is demonstrated that the free hormone fraction f may be considered, like the total hormone concentration, as a function of the free hormone concentration F and binding parameters P_i and K_i only. This will be of much use when dealing with the effects of dilution on the free hormone fraction.

I.4. Free hormone concentration dependence on dilution.

In order to study the effects of changing solution volume, leading either to sample dilution or concentration, eq. (I-6) is extended to include volume changes.
Assume a reference (e.g. undiluted) state for a particular (serum) sample, characterised by binding site concentrations P_{i0} and total and free hormone concentrations of H_0 and F_0.
Then of course:

$$H_0 = F_0 \, \Sigma \frac{P_{i0}}{K_i + F_0} + F_0 \qquad \text{(I-6a)}^{*}$$

* Σ denotes $\sum_{i=1}^{n}$ unless otherwise indicated.

When the sample is brought from an initial volume V_0 to a final volume V_t, the concentrations of binding sites and of total hormone will change by a factor V_0/V_t. Simultaneously, F_0 will attain a new value F:

$$H_0(V_0/V_t) = F \sum \frac{P_{i0}(V_0/V_t)}{K_i + F} + F \qquad \text{(I-12a)}$$

multiplication of both sides by V_t/V_0 gives a general expression for the effect of dilution:

$$H_0 = F \sum \frac{P_{i0}}{K_i + F} + (V_t/V_0)\ F \qquad \text{(I-12b)}$$

of which (I-6a) is a particular case for $V_t = V_0$.
As indicated before, application of equilibrium dialysis will introduce one or more dilution steps which will lead to a change in free hormone concentration for F_0 to F. Since most likely the objective of a free hormone determination is to estimate F_0, which corresponds to the undiluted state of the (serum) sample, rather than F, which has significance only for one particular diluted state, the relation between F_0 and F - more generally represented by the relation between F and dilution factor V_t/V_0 (eq. I-12b) - obviously is very important.
A qualitative picture of the effect of dilution as represented by the dilution factor V_t/V_0 may be obtained by rearranging eq.(I-12b) to:

$$F = \frac{H_0}{\sum \frac{P_{i0}}{K_i + F} + (V_t/V_0)} \qquad \text{(I-12c)}$$

Again, no analytical solution for F as a function of dilution can be found, since F still is present in the right hand member of

(I-12c). However, two limiting conditions can be found, one of which occurs when the dilution factor V_t/V_0 is very small compared with the other term in the denominator. If so, V_t/V_0 may be dropped from the expression (I-12c) for F, so that F becomes independent of dilution and therefore remains constant. An instance where this limiting case holds is described in appendix IA 2.2, where, in the presence of Thyroxine Binding Globulin (TBG), $\Sigma\ P_{10}/(K_1+F)$ is 5000 or higher which causes virtual constancy of free T4 for dilutions up to at least 50 times.

The other limiting condition, leading to a simple relation between F and dilution was already encountered when studying the effect of changes in total hormone, i.e. the condition $F < 0.01\ K_1$ which in the case of dilution is restated as $F_0 < 0.01\ K_1$. This limiting condition is independent of the previous one, so they may hold both separately and simultaneously.

Since it can be proven that $F < F_0$ when $V_t > V_0$, (appendix IC), the condition $F_0 < 0.01\ K_1$ implies that also $F < 0.01\ K_1$. In that case eq.(I-12c) reduces to:

$$F = \frac{H_0}{\Sigma \frac{P_{10}}{K_1} + (V_t/V_0)} \qquad \text{(I-13a)}$$

which represents a simple, though non-linear relation between F and dilution. When (I-13a) is written for the case that $V_t = V_0$,

$$F_0 = \frac{H_0}{\Sigma \frac{P_{10}}{K_1} + 1} \qquad \text{(I-13b)}$$

Subsequent elimination of $\Sigma\ P_{10}/K_1$ makes it possible to express F_0 in F (and also vice versa):

$$F_0 \approx F_0' = \frac{H_0}{H_0/F - V_t/V_0 + 1} \qquad \text{(I-14)}$$

The symbol F_0' was introduced for the following reason:
Expression (I-14), which yields F_0 directly from F when the limiting condition $F_0 << K_1$ holds, may be considered at the same time to represent a general estimator of F_0, irrespective of fulfilment of the limiting condition. It is useful since it provides a better estimate of F_0 than F itself because it can be proven (appendix IC.) that in general $F < F_0' \leqq F_0$.
This estimator also is denoted by F_0' to indicate that it becomes equal to F_0 for the particular case that condition $F << K_1$ holds.
This is the case for aldosterone in serum, where
$F = 1.2 \times 10^{-12}$mol/l and $K_1 = 10^{-6}$mol/l.
In appendix IA.2.1, eq.(I-14) is applied to calculate F for various values of V_t/V_0 from known F_0 and H_0. This relation is also of practical importance since it permits the estimation of the free hormone concentration in the original sample from a value of F measured in an equilibrium dialysate (ch IV.2).

For the simulation of the dependence of F on dilution when the binding parameters (P_1's and K_1's) are known, eq.(I-12b) can be used as such or after simplifications analogous to the operation by which eq.(I-6) was converted into (I-10). This approach is employed in appendix IA.2.2. for examination of the influence of dilution on free T4.

The importance which is experimentally assessible, necessitates examination of its dependence on dilution as well. A general expression for the effect of dilution on the free hormone fraction is derived from the definition f = F/H combined with eq.(I-12c):

$$f = \frac{F}{H} = \frac{F}{H_0 V_0/V_t} = \frac{1}{V_0/V_t \, \Sigma \frac{P_{10}}{K_1 + F} + 1} = \qquad \text{(I-15a)}$$

$$= \frac{V_t/V_0}{\Sigma \frac{P_{i0}}{K_i + F} + V_t/V_0} \qquad \text{(I-15a) continued)}$$

of which the following is a particular case:

$$f_0 = \frac{F_0}{H_0} = \frac{1}{\Sigma \frac{P_{i0}}{K_i + F_0} + 1} \qquad \text{(I-15b)}$$

The consequences of application of the limiting conditions to eqs. (I-15) will be discussed starting with the condition which leads to constancy of F, which also implies $F_0 = F$, and therefore $K_i + F_0 = K_i + F$. This equality permits elimination of all P_i and K_i containing terms from eqs.(I-15), which results in:

$$f_0 \simeq f_0' = \frac{1}{(1/f - 1)V_t/V_0 + 1} \qquad \text{(I-16)}$$

Analogous to the definition of F_0' in eq.(I-14), the parameter f_0' is introduced as an estimator of f_0 to which it is equal only when $K_i + F$ is constant.
The other limiting condition, i.e. F_0, $F < 0.01\ K_1$, also leads to $K_i + F_0 = K_i + F$ for all i, and hence, after the same elimination, to the same expression (I-16). Thus, both limiting conditions result in a non-linear but otherwise simple relation between f and dilution. More insight in this relation is obtained when (I-15a) is re-arranged such, that an expression for the free to bound ratio f/b (= f/(1-f)) is obtained:

$$f/b = f/(1-f) = \frac{V_t/V_0}{\Sigma \frac{P_{i0}}{K_i + F}} \qquad \text{(I-17)}$$

It is evident that the constancy of $K_1 + F$ leads to a direct proportionality between f/b and dilution factor V_t/V_0. This proportionality, which results from either (or both) of the limiting conditions which cause constancy of $K_1 + F$, will be denoted by "linear dilution effect". It has important theoretical and practical consequences, as it forms the basis of the estimation of the free hormone fraction in an undiluted sample from equilibrium dialysis data (ch.IV.2).

Although both limiting cases (F = constant and $F_0 < 0.01\ K_1$) lead to constancy of $K_1 + F$ and hence to eq.(I-16), it must be remembered that still an important difference exists. Indeed, constancy of F must inevitably lead to a direct proportionality between f and dilution since f was defined as the ratio of free to total hormone, where the latter is obviously inversely proportional to dilution. Alternatively, the proportionality of free hormone fraction and dilution may be derived from eq.(I-16) since it can be shown that conditions leading to constancy of F implicitly result in $1/f << 1$ and hence also $(V_t/V_0)(1/f) << 1$, so that (I-16) reduces to:

$$f_0'/f = V_0/V_t \qquad \text{(I-18)}$$

It is concluded that (I-16) represents a relation which holds for either or both limiting conditions whereas (I-18) represents a particular case which holds under the conditions that are consistent with constant F only.

In conclusion it is attempted to give a general description of the dilution effect by making use of the findings in this paragraph and by reference to the worked out examples in appendix IA.
Much depends on the characteristics of the undiluted state. With regard to the proportion of F_0 and K_1 the possibility that $F_0 << K_1$, and the alternative case that this is not so, are considered. In the first case, F will disappear from eq.(I-12c) so that (I-13a) is obtained. It will depend on the relative magnitudes of $\Sigma\ P_{10}/K_1$ and V_t/V_0 whether F will change when diluting. The concentrations of binding sites P_{10} are particularly important in that they determine

at what dilution factor F will begin to change appreciably. Up to this dilution factor, both limiting cases i.e. F = constant and $F << K_1$ hold simultaneously. At higher dilutions only the second one will hold. Anyway, eqs.(I-13) remain valid so that the direct proportionality of f/b and dilution holds from undiluted up to any conceivable dilution factor.

In IA.2.2 such a situation is encountered for free T4 in the absence of TBG. Here $F_0 = 1.875 \times 10^{-11}$ mol/l, while $K_1 = 10^{-8}$ mol/l (TBPA, i.e. Thyroxine Binding Prealbumin). Clearly, F may be omitted from eq.(I-12c). Since $\Sigma P_{10}/K_1 = 1258$, dilution up to a $V_t/V_0 = 12$ is not effective in lowering F. Within this range of dilution evidently both f and f/b are proportional with dilution eq.(I-18). At higher dilution, F will decrease appreciably so that eq.(I-18) does not hold any more, but the proportionality of f/b and dilution will be preserved infinitely.

In appendix IA.2.1. which deals with free aldosterone in serum the binding is so weak that F can not be constant even at very low dilution, so that only the limiting condition $F << K_1$ applies and direct proportionality of f/b but not of f with dilution is observed. If F_0 and one or more of the dissociation constants are of the same order of magnitude, it again depends on the relative magnitudes of F_0 and $\Sigma P_{10}/(K_1+F)$ whether F will remain constant. At dilutions which induce a decrease of F, the proportionality between f and dilution - which may have existed only for a very restricted range of dilutions - is lost, and also the proportionality with f/b. However, when comparing eq.(I-15a) with (I-17) it can be seen that the proportionality with f will be stronger affected than the one with f/b, because of the presence of V_t/V_0 also in the denominator of the former expression. Eventually, as F decreases to very low levels, the limiting condition $F << K_1$ will hold. From that point onwards, the proportionality between f/b and dilution is restored.

In IA.2.2 one has a situation where F_0 (1.871×10^{-11} mol/l) is of the same order of magnitude as K_1 (TBG: 4.4×10^{-11} mol/l). $\Sigma P_{10}/(K_1+F) = 5345$, so F will not change appreciably when diluting up to 50 times. At higher dilutions, F falls progressively and the proportionality of f and eventually f/b with dilution is lost. For the range of dilutions studied, F does not fall below $0.01 K_1$, so

the recurrence of f/b proportionality is not yet observed. It is observed in the next example (IA.2.3) where free cortisol drops already at 10% dilution, but f/b proportionality is almost restored at 500 times dilution where the free hormone has dropped to 6.6 x 10^{-10} mol/l, which is almost negligible compared with K_1 (transcortin: 3.7 x 10^{-8} mol/l).

I.5. The equivalence of dialysis and dilution.

With the knowledge from the previous paragraph about the effects of changes in total hormone concentration and of dilution the purpose is now to examine how distribution of the hormone over two compartments, which are separated by a membrane, affects the free hormone concentration.

When a volume V_0' of a diluted or undiluted serum sample is dialysed against a volume V_d of a solution not containing any binding substances (e.g. saline or buffer) then at equilibrium the concentrations of unbound hormone in both half-cells will be equal, provided that ionic strength is sufficient to prevent the occurrence of Donnan effect. Since some buffer may pass over into the serum compartment as a consequence of osmotic pressure difference, dialysand and dialysate will not retain their original volumes but will occupy volumes V_1 and V_2. The total volume V_t of solvent has not changed so that:

$$V_0' + V_d = V_1 + V_2 = V_t \qquad \text{(I-19)}$$

Let the final (equilibrium) concentration of free hormone be F'. If the dialysand contained a volume V_0 of the undiluted sample, the concentration of its binding sites will have changed from P_{10} via $(V_0/V_0')P_{10}$ to $(V_0/V_1)P_{10}$. The concentration of bound + free hormone in the dialysand, H_1, is found by substitution in (I-6):

$$H_1 = F' \Sigma \frac{(V_0/V_1)P_{10}}{K_1 + F'} + F' \qquad \text{(I-20)}$$

Evidently, total hormone in the dialysate, H_2, equals free hormone:

$$H_2 = F \qquad \text{(I-20a)}$$

The total amount of hormone present in the system is still the same as in the original sample with volume V_0, so:

$$H_0V_0 = H_1V_1 + H_2V_2 \qquad \text{(I-21)}$$

Which can be combined with (I-20) to give:

$$H_0V_0 = V_0F'\Sigma \frac{P_{10}}{K_1 + F'} + V_1F' + V_2F' \qquad \text{(I-22a)}$$

and, since $V_1 + V_2 = V_t$:

$$H_0 = F'\Sigma \frac{P_{10}}{K_1 + F'} + F'(V_t/V_0) \qquad \text{(I-22b)}$$

which is identical to (I-12b), so that F' = F.
This important result shows that application of equilibrium dialysi to a sample with volume V_0, in such a way that the total solution volume of the system becomes V_t, results in the same free hormone concentration as the one that would result when the sample had been diluted directly from V_0 to V_t. Moreover, V_1 and V_2 disappeared from the expression, which demonstrates that F is independent of possible fluid shift during dialysis. The initial dialysand and dialysate volumes V_0' and V_d do not appear in eq.(I-22b) either, which implies that it is unimportant whether the volume V_0 is diluted to V_0' prior to dialysis or not, as long as V_0 represents the amount of the original sample actually present in the system which has a total volume V_t. This may be illustrated as follows:
Suppose that in order to determine free hormone in serum 1 ml of a twofold diluted sample is dialysed against 1.5 ml of buffer. In that case V_0' = 1 ml, V_d = 1.5 ml and V_t = 2.5 ml. Since undiluted

serum is considered as the original sample, V_0 = 0.5 ml so that V_t/V_0 = 5. If instead of 1 ml of the diluted serum, 0.5 ml of the undiluted serum is dialysed, this time against 2.0 ml of buffer, V_0' = 0.5 ml and V_d = 2.0 ml but still V_t/V_0 = 5.

As stated in the introduction to this chapter, the process may be viewed as a decrease of the total hormone concentration in the dialysand as a result of migration of some hormone into the dialysate. An attempt to describe the process in such terms using the result from the very first part of this chapter would be incomplete, since the (possible) concomitant dialysand dilution before or during dialysis also should be taken into account. Therefore, the most straightforward approach is to make use of the equivalence between the equilibrium dialysis process and dilution per se in order to find the relation between observed quantities such as F and the free hormone concentration F_0 in the original sample. However, direct assay of F in the dialysate is often not feasible for lack of sensitivity of the assay method. In such cases the free hormone fraction is determined from the distribution of added radioactive labelled hormone over the two compartments. Therefore also the effect of dialysis on f has to be studied. Definition of a free hormone fraction in a dialysis system must be unequivocal since there are two different total hormone concentrations it could be related to.

One of these, the concentration in the dialysand, depends on chance factors like the amount of solvent that has migrated within a certain time under the influence of osmotic pressure. This difficulty is easily circumvented by relating the total amount of hormone present (i.e. H_0V_0) to the total volume V_t of the system. When the free hormone fraction f for the dialysis system now is defined as the ratio of F, the (unbound) concentration as found in the dialysate, to H_0V_0/V_t, one obtains exactly the same expression as (I-15a) which was derived for mere dilution. Apparently, f, like F is independent of the location of the membrane within the system, and even it does not matter whether it is there at all. Thus, the analogy between equilibrium dialysis and dilution per se with regard to free hormone concentration and fraction is completed.

It must be concluded from the above that application of equilibrium dialysis is virtually impossible without the introduction, to some

extent, of what in fact is a dilution. Consequently, at dialysis equilibrium the free hormone concentration has assumed the value corresponding to this dilution which, as extensively demonstrated, is equal to the original value only in particular cases. As dialysis always involves dilution, the only way to reproduce the original free hormone level in the dialysis system would be by concentrating the sample before dialysis by a factor equal to the "dialysis" dilution factor (Moll et al, 1978). It does not seem feasible to concentrate a serum sample and subsequently dialyse it against the same volume that had been lost by concentrating. However, the principle may be applied to diluted samples. It is for instance possible to measure the true free hormone concentration which corresponds to twofold diluted serum by hormone assay in a dialysate obtained after dialysing undiluted serum against an equal volume of buffer. The "dialysis" dilution factor is 2 for this sample, which is just the dilution factor for which the corresponding free hormone level was required. This principle will be applied later in several instances.

I.6. Discussion.

The mathematical model derived and employed in this study is preferred to the models by e.g. Mills (1962), Tait et al (1964), Oppenheimer et al (1964), Robbins (1967) or Princé et al (1977) because the number of orders of binding sites is unlimited and it contains as variables only free and total ligand concentrations, such in contrast with the other approaches mentioned which are either restricted to a very limited number of orders of sites (Mills, Tait) or in which the concentrations of ligand bound to the different orders of binding sites also appear as variables. In the present model, these concentrations are still discernible as the individual terms under the Σ sign when multiplied by the free hormone (ligand) concentration (eqs.I-4 and I-6). The model is also readily modified to include the dependence on dilution.
The model by Feldman (1972) was derived for m ligands and n orders of binding sites but does not include dilution effects. Yet this model shows more common traits with the present one than any of the

others mentioned. A further difference between the two approaches is the use of the dissociation constants instead of the association constants as also employed by many other authors besides Feldman. There is a distinct advantage in expressing all constants and variables of the binding system in the same dimensions (concentration) because then they may be compared with each other. Thus, the existence of limiting cases for the expression for the concentration of ligand bound to sites of order is much more rapidly visualized and handled when expression (I-4)

$$B_1 = \frac{P_1 F}{K_1 + F} \qquad \text{(I-4)}$$

is used than if the equivalent, using the association constant K_1' is employed:

$$B_1 = \frac{P_1 K_1' F}{1 + K_1' F} \qquad \text{(I-4')}$$

Simplifications of the binding model by means of application of limiting cases has also been applied by others.
The limiting case resulting in proportionality between B_1 and F (eq.I-7a) according to Tait, Oppenheimer and Princé originates from the condition $B_1 << P_1$, i.e. the maximal binding capacity P_1 of an order of sites exceeds by far the amount of ligand actually bound (B_1). It can be readily shown by considering (I-3) and (I-4) that this condition is equivalent to the condition $F << K_1$ as used in this chapter, but the latter is preferred because it is much more general for the following reasons:

1. The concentration of binding sites i, P_1 depends upon the particular sample at hand, whereas K_1 is a constant, characteristic of ligand and binding sites and the same for any sample containing these binding sites.
2. The concentration of unbound ligand F is the net resultant of interaction between total ligand H and the complete set of binding sites present and as such characteristic of the total

binding system.

3. Because the saturation of an order of binding sites i was shown to be a matter of K_i and F only, the use of B_i vs. P_i as a criterion wrongly suggests that P_i is the primary factor in determining the saturation of binding sites i. In fact, the presence of other binding sites also influences F and thereby B_i and it might be even so that the concentration of a particular order of binding sites plays a negligible role in determining its own saturation.

By comparing the value of F with the various K_i's it is possible to find out for what values of F which non-linear terms in the binding equation may be replaced by linear ones. Use of the B_i vs. P_i criterion requires calculation of B_i's first from the general, unreduced expression.

Application of the model for studying the effect of changes in total hormone on free hormone levels has its main interest in the study of the significance of the hormone binding proteins in blood, as is illustrated by the fact that in the litterature mathematical models are applied mainly for that purpose. In a few instances, however, mathematical models for hormone interaction with multiple binding proteins were applied in an attempt to determine free hormone in undiluted serum from equilibrium dialysis measurements performed in the diluted state (Oppenheimer et al. 1964, Vermeulen et al. 1971). Since it was demonstrated that the complete equilibrium dialysis process, including sample dilution before dialysis, is most conveniently described as one dilution process, the latter application has more relevance for the present study. However, the relation between total and free hormone is dealt with rather extensively both in theory and in worked out examples for its illustrative power and in order to demonstrate that the present model is also very well suited for simulation studies of more physiological interest.

The effect of dilution on free hormone concentration can not be very simply described for a general case, although the limiting conditions given in I.4. are of much help.

The effect of dilution on free hormone was theoretically derived

by Oppenheimer (1964) for the particular case of T4 in normal human serum. Although a cumbersome derivation was applied which resulted from not using limiting conditions based on comparison of free hormone concentrations and dissociation constants, this author arrives at largely the same result concerning the constancy of free T4 for a relatively large dilution range as was obtained in the present report (see IA.2.2.). However, the proporrionality between f/b and dilution which holds for a considerably longer dilution range remained unnoticed. Therefore Oppenheimers consideration remains restricted to normal and high TBG serum. The constancy of free T4 thus made plausible was applied by Oppenheimer to calculate free T4 in undiluted serum from measurements of the free hormone fraction carried out by equilibrium dialysis of diluted serum.

The simple constancy of free hormone on dilution does not hold for free testosterone in serum. Vermeulen (1972) derived a formula by which free testosterone in undiluted serum can be calculated from total and free measurements made in the diluted state and known values for the dissociation constants of the most important binding proteins. This - also valid - approach was necessitated by the fact that no simple relation between free testosterone and dilution could be found. Instead of making use of a model for hormone binding to multiple binding sites, also purely empirical methods have been applied (Kley et al. 1977). Free hormone fraction measurements were done at different dilutions and the free fraction for undiluted serum was obtained by extrapolation.

In any of the cases mentioned it is necessary that the dilution at which measurements are made is defined in an unequivocal way. Very correctly, Oppenheimer considered the ratio of the volume of undiluted serum present to the total volume (dialysate + dialysand) of the system as the dilution factor which corresponds to the measurement. Although not stated explicitly, this means that this author recognised the equivalence of dialysis and dilution. Also Ekins (1975) pointed out that the ratio of serum volume to the total volume in the dialysis system constitutes the true dilution factor.

In contrast, both Vermeulen and Kley consider the dilution factor in the dialysand as representative of the state of the sample in

which the actual measurement is made. Consequently, these authors - as well as many others - seek to maintain the concentration of the sample in the dialysand at a constant level during dialysis by diluting the sample prior to dialysis so that no noticeable fluid shift caused by osmotic pressure leading to dilution in the dialysand, will occur.
It was demonstrated in I.5. that fluid shifts within the system do neither affect the free hormone concentration, nor the free hormone fraction, if properly defined.
The method for assessment of the free hormone fraction generally used (Slaunwhite et al. 1959) does not yield this free hormone fraction which belongs to the "total" dilution of the sample. Instead it gives the actual free hormone fraction in the dialysand which corresponds to and depends on the dilution within that compartment. It is commonly mistaken as representative also of the original sample when diluted to the same degree. This would only be the case if the "linear dilution effect" holds.

When one realises that the free hormone concentration is determined only by the ratio of the volume of the serum in the system, V_0, and the total volume of the dialysis system, V_t, the possibility of assessment of free hormone without any of the problems caused by dialysand dilution, opens (ch. IV.2.).
One other effect that dilution may have on the hormone-protein equilibrium, although not included in the mathematical model employed here, deserves mentioning. If inhibitors (or activators) of binding are present, their effect is expected to decrease progressively upon dilution if they are less strongly protein bound than the hormone. This may, in case of inhibitors, result in an actual lowering of the free hormone concentration when diluting also in situations where binding is so strong that constancy of F is anticipated for a considerable range of dilutions. This effect is more extensively discussed in ch. V.4.2.

I.7. Summary

In this chapter a mathematical model for the interaction between

hormone and serum binding proteins is derived (I.2.). This model is primarily meant for investigating the effect of application of equilibrium dialysis on the free hormone concentration and its consequences for the interpretation of experimental data.
First the mathematical model is applied for examination of the influence of total on free hormone concentrations (I.3.). In this model each class of binding sites present contributes a certain concentration of hormone bound to it to the total hormone concentration. These individual contributions are generally a non-linear function of the free hormone concentration, but they reduce to simple, linear ones under certain limiting conditions, thus leading to a simple expression for total hormone. These limiting conditions are for each order of binding sites defined in terms of the relative magnitude of free hormone concentration and dissociation constant. When the former is either extremely small or extremely large as compared to the latter, the above simplification may be applied for that order of sites.
In the examination of the effect of dilution on free hormone concentration (I.4.) also the occurence of limiting cases is employed. In addition to comparing free hormone concentration with dissociation constants, also a comparison of the dilution factor with the strength of binding may lead to detection of simpler relationships than the general one. It is found that, when for undiluted serum the free hormone concentration is much smaller than all dissociation constants, a direct proportionality exists between the ratio of free to bound hormone and dilution.
In I.5. it is demonstrated that the application of equilibrium dialysis to a serum sample has the same effect on the free hormone concentration as diluting that sample to a volume equal to the total volume of dialysand + dialysate. Fluid shift between dialysand and dialysate has no influence. The only determining factor is the ratio of the volume of serum present in the system (V_0) and the total volume of the dialysis system (V_t). This result forms the basis of methods for evaluation of equilibrium dialysis data which are independent of dialysand dilution.
In the appendix examples concerning the relation between free hormone and total hormone and dilution that are based on actual situations (free aldosterone, thyroxine and cortisol in serum) are

worked out as an illustration of the possible forms of the interrelations between free hormone and total hormone and dilution. This is considered to be useful for a better understanding of the methods for evaluating free hormone concentrations from equilibrium dialysis data but also to demonstrate the usefulness of the binding model for physiological study.

Appendix IA.

The influence of changes in total hormone and dilution on free hormone concentration: Simulation studies (worked out examples).

IA.1. The effect of changes in total hormone.

IA.1.1. Free aldosterone.

IA.1.2. Free thyroxine.

IA.2. The effect of dilution.

IA.2.1. Free aldosterone.

IA.2.2. Free thyroxine.

IA.2.3. Free cortisol.

For a better understanding of the interrelationships between free and total hormone levels and solution volume, values of these variables are calculated according to the mathematical model and its simplifications as described in this chapter. All kinds of relationships discussed are represented in the worked out examples, which refer to actual conditions since literature values for binding parameters of hormones in serum were used.

IA.1. The effect of changes in total hormone.

IA.1.1. Free aldosterone.

Under normal conditions the total aldosterone concentration in plasma is about 3×10^{-11} mol/l with a corresponding free aldosterone level of 1.2×10^{-11} mol/l. What effect would an increase of total aldosterone have on the concentration of free aldosterone, when it is given that of the aldosterone binding proteins in plasma the corticosteroid-binding globulin (CBG) displays the highest affinity with a dissociation constant $K_1 = 10^{-6}$ mol/l towards this hormone.

From the values for K_1 ($= 10^{-6}$ mol/l) and F ($= 1.2 \times 10^{-11}$ mol/l) it follows that the simplified expression (I-7b) may be used so that for the value $H = 3 \times 10^{-11}$ mol/l one obtains $\Sigma\ P_1/K_1 = 1.5$ from this expression.

This value of $\Sigma\ P_1/K_1$ can be used again to calculate H, correspon-

ding to the maximum value of F (10^{-8} mol/l) for which (I-7b) is still valid. This calculation results in H = 2.5 x 10^{-8} mol/l. So, between the normal level of 3 x 10^{-11} up to 2.5 x 10^{-8} mol/l of total aldosterone the free aldosterone level is directly proportional to total aldosterone, with a constant ratio (= free fraction = F/H) of 0.4.
The above demonstrates that in order to judge whether the simple expression (I-7b) may be applied for description of the relation between free and total hormone, one only needs to know the magnitude of the dissociation constant of the order of binding sites with the highest affinity. The other binding sites present can be ignored in this respect.

IA.1.2. Free thyroxine.

Thyroxine (T4) is bound in human serum mainly by three binding proteins: Thyroxine Binding Globulin (TBG), Thyroxine Binding Pre-Albumin (TBPA) and serum albumin (HSA). The concentrations of the different orders of binding sites and their corresponding dissociation constants are (data from Wosilait et al. 1977):

P_1: 0.254 x 10^{-6} mol/l, K_1 = 4.4 x 10^{-11} mol/l (TBG)
P_2: 3.97 x 10^{-6} mol/l, K_2 = 1 x 10^{-8} mol/l (TBPA, 1st site)
P_3: 580 x 10^{-6} mol/l, K_3 = 7.7 x 10^{-7} mol/l (HSA, 1st site)
P_4: 3.97 x 10^{-6} mol/l, K_4 = 1.05 x 10^{-6} mol/l (TBPA, 2nd site)
P_5: 1740 x 10^{-6} mol/l, K_5 = 1.67 x 10^{-5} mol/l (HSA, 2nd order of 3 identical sites)

The values of total T4 for which the influence of change in total hormone on free hormone will be evaluated range from 10^{-9} to 10^{-5} mol/l, which means from 100 x lower up to 100 x higher than the normal mean total T4 of 100 nmol/l (normal range: 60 - 150 nmol/l). The general expression for total T4 as a function of free is:

$$H = F \sum_{i=1}^{5} \frac{P_i}{K_i + F} + F \qquad \text{(IA-1)}$$

This relation permits direct calculation of total T4 (H) for given values of free T4 (F). For the inverse operation, calculation of F for known values of H, no algebraic solution can be found so one must resort to a numerical one. From the given K_1's it can be seen that for free T4 concentrations below 7.7×10^{-9} mol/l (= 0.01 x K_3) the concentration of the hormone bound to sites of the order 3, 4 and 5 (B_3, B_4 and B_5) can be written in the linear form of (I-7) so that for $F < 7.7 \times 10^{-9}$ mol/l eq.(IA-1) reduces to:

$$H = F \sum_{i=1}^{2} \frac{P_i}{K_i + F} + F \sum_{i=3}^{5} \frac{P_i}{K_i} + F \qquad \text{(IA-2)}$$

Substitution of the limiting value of F (7.7×10^{-9} mol/l) in eq.(IA-1) gives the corresponding total hormone concentration $H = 8.56 \times 10^{-6}$ mol/l. Up to this value, which encloses the normal range, eq.(IA-2) forms a sufficiently accurate approximation of eq.(IA-1) so that it may be safely applied for simulation of the relation between free and total hormone for total T4 ranging from $0 - 8.56 \times 10^{-6}$ mol/l.

Evaluation of expressions like (IA-2), in which for only two orders of binding sites the general formula is used and binding to the other sites is simply summed, is still relatively simply carried out on a programmable pocket calculator. Above a total T4 level of 8.56×10^{-6} mol/l also for the third order of binding sites the non-linear form has to be used and a more complex expression is obtained. Fortunately then the other limiting case can be applied for the first class, namely for a concentration of free T4 from 4.4×10^{-9} mol/l (= 100 K_1) onwards.

For this value, the TBG binding sites are virtually completely occupied so the simpler form of (I-10) may be used, resulting in:

$$H = P_1 + F \sum_{i=2}^{3} \frac{P_i}{K_i + F} + F \sum_{i=4}^{5} \frac{P_i}{K_i} + F \qquad \text{(IA-3)}$$

This expression may be used between values of F of 4.4×10^{-9} mol/l (= 100 K_1) and 1.05×10^{-8} mol/l (= 0.01 K_4) which corresponds to total T4 values of 5.24×10^{-6} and 1.10×10^{-5} mol/l, as calculated

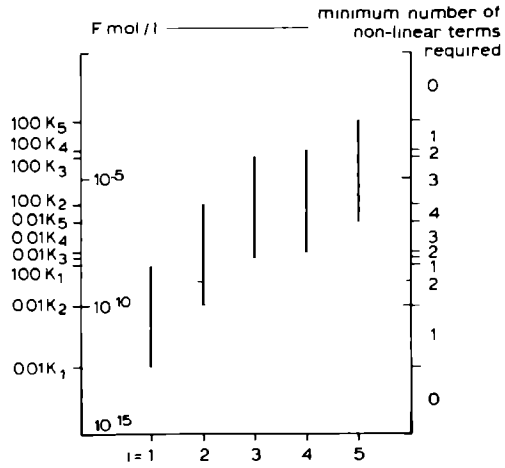


Fig.IA.1.

Diagram representing the minimum number of non-linear terms required in the binding equation in dependence of free hormone concentration. Vertical bars indicate for any of the five orders of T4 binding sites present (appendix IA.1.2.) the range of values of F (from 0.01 K_1 up to 100 K_1) where the general, non-linear notation for the concentration of bound hormone B_1 must be used. By drawing horizontal lines, for any value of F the notation yielding maximal simplification can be found. E.g. at a value of F of 10^{-9} mol/l (dotted horizontal line) the first two sites require non-linear notation. For the other three sites $F < 0.01\ K_1$ so that for B_1 (i= 3, 4, 5) the linear notation according to eqs. (1-7) may be used.

with help of (IA-1).

For solution of the problem, values of F are calculated for total T4 from 10^{-9} up to 5 x 10^{-6} mol/l using (IA-2) and for total T4 between 5 x 10^{-6} and 10^{-5} mol/l (IA-3) is used. Further subdivision is possible (fig. IA.1) since for F < 10^{-8} mol/l (0.01 K_2) only binding to TBG has to be expressed in the non-linear form. However, this is of little use since eq.(IA-2) which includes this particular case, can be handled quite adequately.

A complete account of simplifications that may be applied at all is given in fig. IA.1, while table IA.1 lists the values of F (T4) calculated by means of eq.(IA-2) using both the binding parameters and the total hormone levels from the report by Wosilait (1977) and compares these with the corresponding values in the original article, which were obtained by computer evaluation using a formula essentially equal to (IA-1).

The complete set of data produced in that report, including the concentrations of T4 bound by the various protein fractions, can be obtained in a few hours using a programmable (32 steps) pocket calculator (see appendix IB).

total T4 (nmol/l)	free T4 (pmol/l)	
	from Wosilait (1977)	present method
12.8	1.87	1.865
38.6	6.04	6.041
64.3	10.8	10.83
100	18.7	18.71
154	33.9	33.91
192	47.4	47.35

Table IA.1.

Free T4 levels from binding parameters and total T4 data from Wosilait (1977) as calculated in the original report by means of an IBM 370/168 computer requiring in total 444 K bytes for carrying out the program and by means of the present method using the simplified expressions and calculations, on a Texas Instruments 51-III pocket calculator with 32 program steps. (Program: see appendix IB)

Total T4 mol/l	Normal TBG (0.254×10^{-6} mol/l)				High TBG (0.5×10^{-6} mol/l)				Absent TBG	
	eq.(IA-2)		eq.(IA-3)		eq.(IA-2)		eq.(IA-3)		eq.(IA-2)	
	F (T4) mol/l	f (T4)	F (T4) mol/l	f (T4)	F (T4)	f (T4)	F (T4) mol/l	f (T4)	F (T4) mol/l	f (T4)
10^{-9}	1.412×10^{-13}	1.412×10^{-4}			7.850×10^{-14}	7.850×10^{-5}			7.936×10^{-13}	7.936×10^{-4}
2×10^{-9}	2.831	1.416			1.572×10^{-13}	7.862			1.587×10^{-12}	7.936
5×10^{-9}	7.135	1.427			3.951	7.901			3.968	7.937
10^{-8}	1.446×10^{-12}	1.446			7.966	7.966			7.937	7.937
2×10^{-8}	2.973	1.486			1.620×10^{-12}	8.099			1.588×10^{-11}	7.939
5×10^{-8}	8.083	1.617			4.261	8.523			3.973	7.946
10^{-7}	1.871×10^{-11}	1.871			9.320	9.320			7.956	7.956
2×10^{-7}	5.049	2.524			2.268×10^{-11}	1.134×10^{-4}			1.595×10^{-10}	7.976
5×10^{-7}	2.290×10^{-10}	4.580			1.116×10^{-10}	2.232			4.018	8.036
10^{-6}	6.166	6.166	6.031×10^{-10}	6.031×10^{-4}	4.383	4.383			8.133	8.133
2×10^{-6}	1.449×10^{-9}	7.246	1.445×10^{-9}	7.223	1.247×10^{-9}	6.236			1.664×10^{-9}	8.320
5×10^{-6}	4.152	8.304	4.165	8.331	3.924	7.847	3.933×10^{-9}	7.865×10^{-4}	4.408	8.815
10^{-5}	9.100	9.100	9.184	9.184	8.849	8.849	8.926	8.926	9.451	9.45

Table IA.2.

Free T4, F(T4) and free T4 fraction f(T4) as a function of total T4. Binding parameters from Wosilait (1977), except for high and absent TBG. The range of free T4 values requires the use of two forms of expression (I-10), namely (IA-2) and (IA-3). except in the case of TBG deficiency.

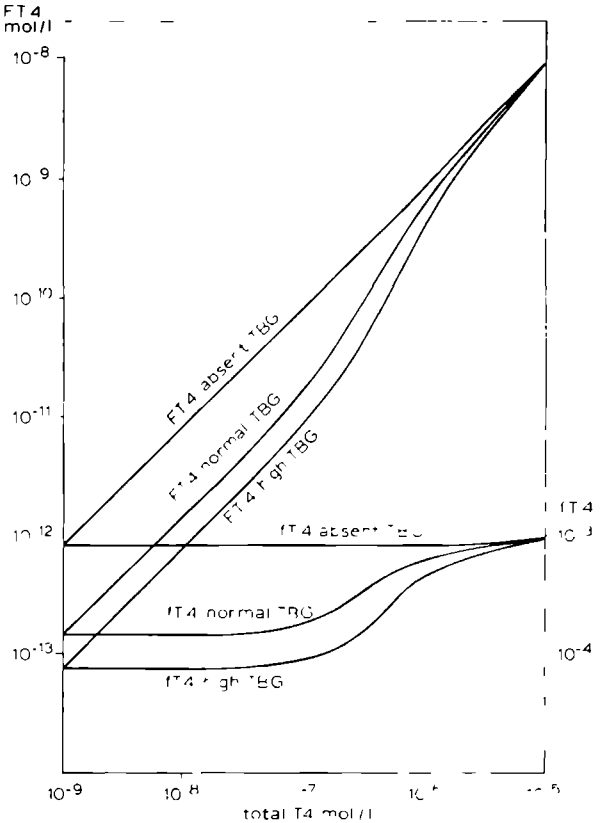


Fig. IA.2.

Graphical representation of data from table IA.2.

nmol/l	F(T4) mol/l	f(T4)	F(T4) mol/l	f(T4)	F(T4) mol/l	f(T4)
0*	0	1.426×10^{-4}	0	7.837×10^{-5}	0	7.935×10^{-4}
25	3.819×10^{-12}	1.528	2.042×10^{-12}	8.167	1.985×10^{-11}	7.940
50	8.200	1.640	4.261	8.523	3.973	7.945
75	1.323×10^{-11}	1.764	6.679	8.906	5.963	7.950
100	1.901	1.901	9.320	9.320	7.956	7.956
125	2.563	2.051	1.221×10^{-11}	9.767	9.951	7.961
150	3.319	2.212	1.538	1.025×10^{-4}	1.195×10^{-10}	7.966
175	4.175	2.386	1.886	1.078	1.395	7.971
200	5.136	2.568	2.269	1.134	1.595	7.976
225	6.203	2.757	2.690	1.196	1.796	7.981
250	7.375	2.950	3.156	2.262	1.996	7.986

Table I A 3.

Values for free thyroxine (F(T4), mol/l) and the free thyroxine fraction (f(T4)) for three different concentrations of TBG as a function of total thyroxine concentration within the patho-physiological range on a linear scale.

* Values for f(T4) corresponding to TT4 and FT4 = 0 must be considered as $\lim_{F \to 0} f(T4)$

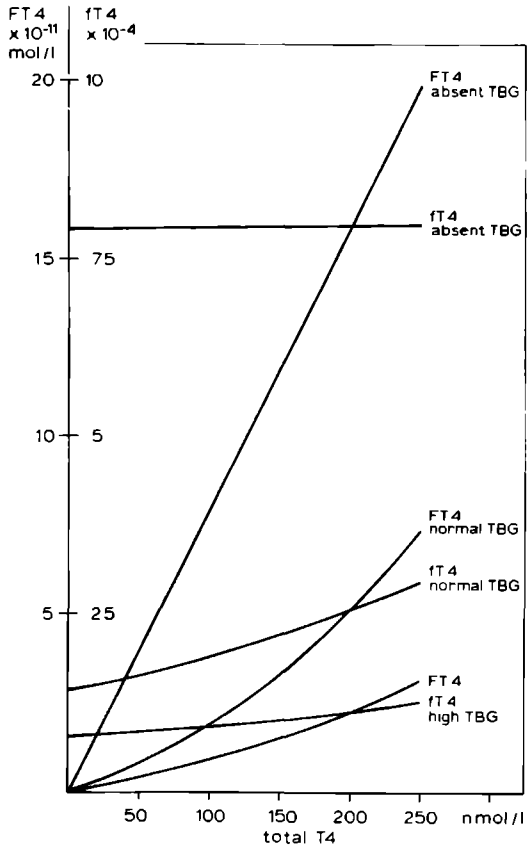


Fig. IA.3.

Graphical representation of data from table IA.3.

Table IA.2 gives the values of F and f calculated by means of eqs. (IA-2) and IA-3) for three different cases: normal TBG (P_1 = 0.254 x 10^{-6} mol/l i.e. the same as in Wosilait's report) high TBG (P_1 = 0.5 x 10^{-6} mol/l, i.e. a pregnancy value) and absent TBG. In the latter case eq.(IA-2) was applied with the first binding sites of TBPA now taken as P_1. F (T4) values were calculated first and corresponding free T4 fractions were obtained by: f(T4) = F(T4)/ total T4. The data from table IA.2 are represented on a logarithmic scale in fig. IA.2. For the range of total T4 concentrations of patho-physiological interest (0 - 250 nmol/l) an additional set of data was produced (table IA.3) which is graphically represented in fig. IA.3.

In the aldosterone example the binding proteins merely provide a proportional relationship between total and free hormone, whereas in the case of T4 such a simple relation exists only in the absence of TBG. A possible physiological significance (although not yet understood) of the presence of TBG becomes apparent in the normal range of total T4 (60 - 150 nmol/) where an increase in total T4 is accompanied by an increase in the free hormone fraction as a result of increasing TBG saturation, so that the effect of a rise in total T4 on free T4 is amplified (table IA.2 and fig. IA.2). This rise in f can be understood with help of eq.(I-11). Eq.(I-11) shows that increases in F which necessarily accompany a rise in total hormone, lead to an increase in f too, provided that the increment of F is significant as compared with K_1. This will be the case for TBG ($K_1 = K_1$ = 4.4 x 10^{-11} mol/l) already in the normal range for free T4 (1.0 - 2.5 x 10^{-11} mol/l) as shown in figs. IA.2 and 3. In the absence of TBG the lowest K is that of the prealbumin, i.e. 10^{-8} mol/l. Consequently, a rise of F will be discernible only at values of F > 10^{-10} mol/l.

After TBG has been saturated, the relative contribution of TBG-bound T4 to total hormone will fall as total hormone rises and the F vs H as well as the F vs H curves for the different TBG concentrations including the zero level eventually will coincide (fig. IA.2).

IA.2. The effect of dilution.

IA.2.1. Free aldosterone.

In IA.1.1 it was shown that F_0 (1.2×10^{-11} mol/l) < 0.01 K_1 (10^{-8} mol/l) and that this inequality holds for all other orders of binding sites as well. Since it can be demonstrated (appendix IC) that $F < F_0$ when $V_t > V_0$ the inequality will remain valid for all orders of sites also when the serum is diluted. Consequently, eq.(I-14) may be applied. Since in this instance the purpose is to calculate values for F corresponding to different dilutions, with F_0 known, eq.(I-14) is rearranged to:

$$F = \frac{H_0}{H_0/F_0 + V_t/V_0 - 1} \qquad \text{(I-14a)}$$

Values of F as a function of V_t/V_0 are listed in table IA.4 and graphically represented in fig. IA.4. Included are also the corresponding values of f, calculated from $f = FV_t/H_0V_0$, and f/b which is found from f, since f/b = 1/(1/f-1). Known values of F_0 and H_0 from IA.1.1 (1.2×10^{-11} and 3×10^{-11} mol/l) were used in the computatations.

The shape of the observed curves for F, f and f/b is readily explained by means of eqs.(I-13a), (I-15a) and (I-17). It was already shown that $F << K_1$ holds for all binding sites $i = 1.....n$ and all F obtained by dilution, so that (I-12c), which represents the general relation between F and dilution, reduces to (I-13a).

In IA.1.1, $\Sigma P_{i0}/K_1$ was found to be 1.5, which by no means is high enough to render V_t/V_0 in (I-12c) negligible, so that F can not be constant. Therefore, a non-linear decrease of F according to (I-13a) and (I-14a) is observed. Still, the same fact that $F << K_1$, causes both a direct proportionality of f/b and V_t/V_0 according to eq.(I-17) and the disappearance of F from the denominator of (I-15a) so that a simple, though non-linear relation between f and dilution is obtained.

V_t/V_0	F x 10^{-11} mol/l	f	f/b
1	1.20	0.40	0.669
2	0.86	0.57	1.33
5	0.46	0.77	3.35
10	0.26	0.87	6.69
20	0.14	0.93	13.3
50	0.058	0.97	33.5
100	0.030	0.98	66.9
200	0.015	0.99	133
500	0.006		335
1000	0.003		669

Table IA.4.

Values for free aldosterone (F), free aldosterone fraction (f) and free to bound ratio f/b as a function of dilution factor V_t/V_0, calculated with help of eq.(I-14a)(appendix IA.2.1.)

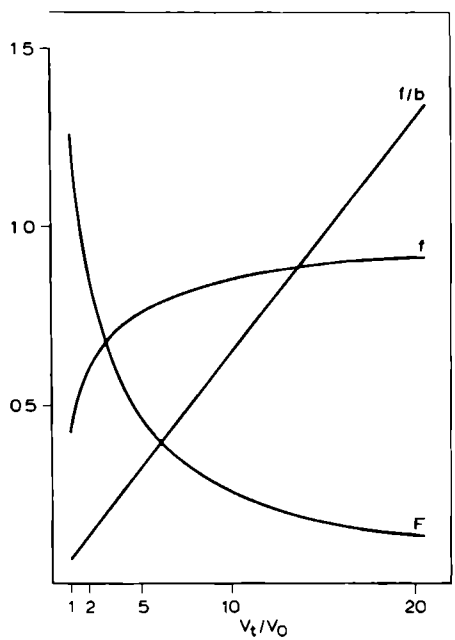


Fig.IA.4.

Representation of data from table IA.4.
Free aldosterone fraction (f) against dilution factor. For the free aldosterone concentration and the free/bound ratio, the vertical scale must be multiplied by 10^{-11} mol/l and 10 respectively.

IA.2.2. Free thyroxine.

Simplifications like the one described in the aldosterone example cannot be applied for T4 in serum, since the condition $F << K_1$ is certainly not fulfilled for any order of binding sites present. Again an iteration method must be used, for which (I-12) is re-arranged to obtain an expression for the dilution factor:

$$V_t/V_0 = H_0/F - \Gamma \frac{P_{10}}{K_1 + \Gamma} \qquad \text{(I-12d)}$$

This general expression may be simplified according to the same principles as applied in IA.1.2. When for H_0 10^{-7} mol/l is taken (an average normal value) the corresponding F_0 is found to be 1.87×10^{-11} mol/l (table IA.2) for serum with a normal TBG concentration. The same initial value for F was chosen for the high TBG and absent TBG samples, corresponding to total T4 values of 1.74×10^{-7} and 2.36×10^{-8} mol/l respectively (see table IA.5). For this value of F_0, only the TBG term has to be written in non-linear form, as can be read from fig. IA.1. This number of terms will not be increased, since F can not rise upon further dilution. Accordingly (I-12d) reduces to:

$$V_t/V_0 = H_0/F - \frac{P_{1(0)}}{K_1 + F} - \sum_{i=2}^{5} \frac{P_i}{K_i} \qquad \text{(IA-4)}$$

Values for F, f and f/b are listed in table IA.5 and graphically represented in figs. IA.5 and IA.5a.
The relative constancy of F found in all three cases can be explained readily by means of eq.(I-12c). The summed terms occurring next to the dilution factor in the denominator of the expression can be calculated for the chosen value for F_0 and appear to be 5345, 9302 and 1258 for the normal, high and zero TBG samples so that dilution factors of 53, 93 and 12.6 contribute only 1% to the magnitude of the expression for F. This implies a long range of constancy of free T4 and therefore of direct proportionality between dilution and the

dilution factor V_t/V_0	Normal TBG: 0.254×10^{-6} mol/l; total T4: 1×10^{-7} mol/l			High TBG: 0.5×10^{-6} mol/l; total T4: 1.740×10^{-7} mol/l			Absent TBG: total T4: 2.356×10^{-8}		
	F (T4) mol/l	f	f/b	F (T4) mol/l	f	f/b	F (T4) mol/l	f	f/b
1	1.871×10^{-11}	1.871×10^{-4}	1.871×10^{-4}	1.871×10^{-11}	1.075×10^{-4}	1.075×10^{-4}	1.871×10^{-11}	7.940×10^{-4}	7.946
2	1.871	3.742	3.743	1.871	2.150	2.150	1.870	1.587×10^{-3}	1.590×10^{-3}
5	1.869	9.348	9.357	1.870	5.374	5.350	1.865	3.958	3.974
10	1.867	1.867×10^{-3}	1.870×10^{-3}	1.869	1.074×10^{-3}	1.075×10^{-3}	1.858	7.884	7.974
20	1.863	3.726	3.740	1.866	2.145	2.150	1.843	1.564×10^{-2}	1.589
50	1.849	9.247	9.333	1.858	5.339	5.368	1.801	3.822	3.974
100	1.828	1.828×10^{-2}	1.862×10^{-2}	1.844	1.060×10^{-2}	1.071×10^{-2}	1.735	7.363	7.948
200	1.786	3.571	3.703	1.819	2.091	2.136	1.616	1.372×10^{-1}	1.590×10^{-1}
500	1.673	8.365	9.129	1.746	5.019	5.284	1.340	2.843	3.972
1000	1.518	1.518×10^{-1}	1.790×10^{-1}	1.640	9.426	1.041×10^{-1}	1.043	4.427	7.944

Table IA.5.

Free thyroxine (F(T4)). Free thyroxine fraction f, and free to bound ratio f/b as a function of dilution, for three different concentrations of TBG.
Total T4 values in the undiluted state correspond to: 7.77; 13.5 and 1.8 µg/100 ml respectively.

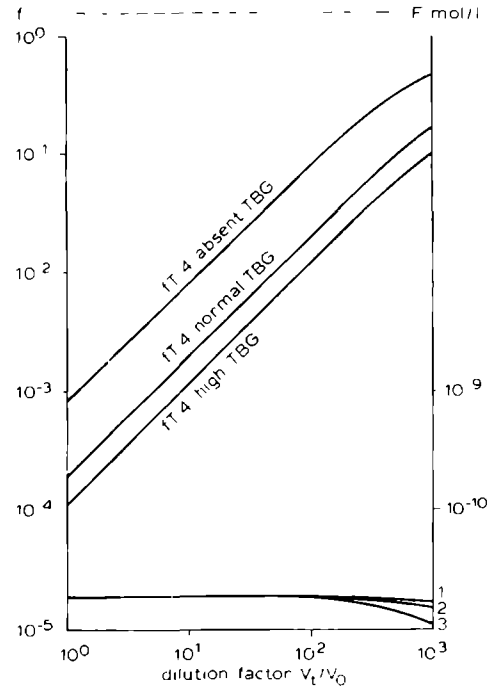


Fig.IA.5.

Free T4 (F, mol/l, lower curves;
1: normal TBG, 2: high TBG, 3: absent TBG)
and free T4 fraction (f, upper curves: fT4)
as a function of dilution factor V_t/V_0.
Data from table IA.5.

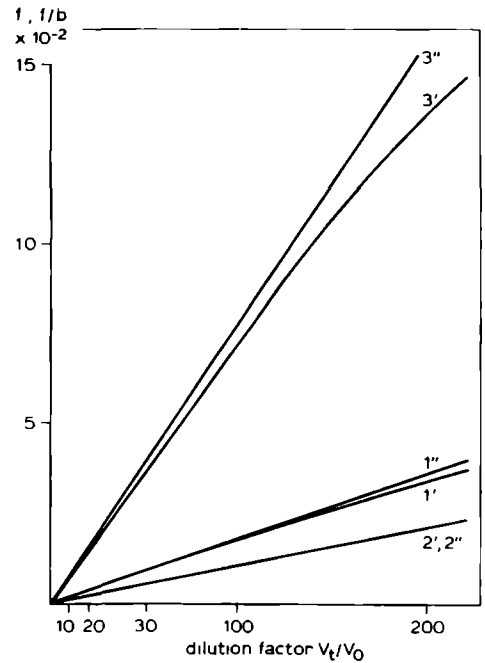


Fig.IA.5a.

Free T4 fraction (f) and free/bound ratio
(f/b) for normal TBG (curves 1' and 1"), high
TBG (curves 2' and 2") and absent TBG
(curves 3' and 3").

free T4 fraction. At higher dilutions a significant contribution of the dilution factor to the denominator will result in progressive lowering of F (eq.I-12c) and therefore in loss of the proportionality between f and dilution. However it will depend on the relative magnitudes of F and K_1 whether the proportionality between f/b and dilution will remain or not.

It is very interesting in this respect to compare by means of table IA.5 the effects of dilution for the three samples differing in TBG content. The relative decrease in F when diluting 1000 times is more pronounced in the normal TBG than in the high TBG sample (23% and 14% respectively), which can be attributed to the different values the first term in the denominator of (I-12c) assumes for both cases i.e. (5345 and 9302 at F_0). This fall in F has a small but distinct influence on the proportionality between f/b and dilution as can be understood from eq.(I-17), where the change in F is significant as compared with the dissociation constant of TBG which belongs to the quantitatively important term representing the ratio of TBG bound to free hormone. In the absence of TBG a marked decrease of free T4 is observed when diluting 1000 fold (79%) as a consequence of the fact that the dilution factor contributes more to the denominator of (I-12c) than in the other two cases. However, this rather massive decrease does not influence whatsoever the proportionality between f/b and dilution, which can be explained by the fact that this change in F still is negligible as compared with the dissociation constant of the highest affinity site which is the prealbumin (first site) with $K = 10^{-8}$ mol/l. These observations are a reflection of the fact that the proportionality between f/b ratio and dilution is of general validity when the condition $K_1 + F$ = constant, is fulfilled whereas the linearity between f and dilution requires that the additional condition $V_t/V_0 << \Sigma P_{10}/(K_1 + F)$ in (I-15a), which leads to almost constant F, is fulfilled and therefore has less general applicability. This also can be demonstrated by calculating F_0' by means of (I-14) and comparing the obtained values for each dilution with those of F from table IA.5. These values for F can be thought of as obtained by direct assay or as determined by assay of f first and subsequent estimation of f_0 by (I-18) (i.e. simple division by the dilution

factor) followed by multiplication with total hormone in the undiluted state (H_0). The values of F_0' (table IA.6, second column) can be obtained in practice by direct application of (I-14) on measured values of F or alternatively by application of (I-16) on measured f, which gives f_0', followed by multiplication by H_0.

Dilution factor	F(T4) mol/l x 10^{11}	F_0'(T4) mol/l x 10^{11}
1	1.871	1.871
2	1.871	1.871
5	1.869	1.870
10	1.867	1.870
20	1.863	1.870
50	1.849	1.868
100	1.828	1.862
200	1.786	1.852
500	1.673	1.825
1000	1.518	1.789

Table IA.6.

Comparison of values of F and F_0' for T4 in serum at increasing dilution. The values of F may be regarded as estimates of F_0 using the assumption that F is independent of dilution, which is the same as assuming that f is directly proportional to dilution (eq.I-18), whereas F_0' values are estimates obtained by application of (I-14) on F or (I-16) on f, which implies the assumption that f/b is directly proportional to dilution.

IA.2.3. Free cortisol.

IA.2.1 and 2 deal with cases in which the effect of dilution on free hormone concentrations and fractions can be described by simple relations. This possibility occurs since either the condition $F << K_1$ holds (aldosterone and T4 in the absence of TBG) or the condition $F = F_0$ (T4 both in the presence and absence of TBG, the latter being an example of validity of both limiting cases for a certain range of dilutions). With regard to cortisol in serum, neither of these conditions holds which is readily demonstrated by means of binding data from the literature:

$K_1 = 3.7 \times 10^{-8}$ mol/l (transcortin)
$P_1 = 5.4 \times 10^{-7}$ mol/l $P_1/K_1 = 0.9$ (mostly albumin)
$H_0 = 3.4 \times 10^{-7}$ mol/l
$F_0 = 3.7 \times 10^{-8}$ mol/l

Since $F_0 = K_1$, no "linear dilution effect" can originate from very low F. The other possible cause for such an effect is (almost) constancy of F when binding is so strong that the dilution factor is negligible in the expression for F (I-12c). By substitution of the above data in (I-12c) it is readily demonstrated that this is not the case either. The term $\Sigma P_{i0}/(K_1 + F_0)$ becomes 8.2 so that V_t/V_0 never will become negligible since its lowest value is 1. The effect of dilution on the above cortisol binding system is readily calculated using an expression of the same form as (IA-4). The results are summarized in table IA.7, which shows that only for very high dilutions (i.e. low F as compared with K_1) the ratio f/b and dilution become proportional. This is most clearly demonstrated by the values of F_0'. The non-linearity of the dilution effect is less pronounced here than with higher total cortisol values. In ch. IV experimental data are given which are representative of the latter case.

Dilution factor	F(cortisol) nmol/l	F_0' nmol/l	f	f/b
1	36.9	36.9	0.109	0.122
2	31.5	34.8	0.186	0.228
5	22.8	31.1	0.335	0.503
10	16.1	28.2	0.475	0.904
20	10.5	25.6	0.620	1.63
50	5.34	23.2	0.786	3.66
100	2.97	22.1	0.874	6.94
200	1.58	21.4	0.931	13.4
500	0.660	20.9	0.970	32.8
1000	0.335	20.8	0.985	65.1

Table I A.7

The effect of dilution on free cortisol concentration and related parameters as calculated from the data given in appendix IA.2.3. The values for F and f demonstrate that no proportionality between the free hormone fraction and dilution exists whereas the data for F_0' and f/b show that the "linear dilution effect" occurs only at very high dilutions, i.e. at very low F.

Appendix IB.

A calculator program for evaluation of free hormone dependence on total hormone and dilution.

With the Texas Instruments TI-51-III programmable pocket calculator it is possible to evaluate the free hormone concentration F for varying values of the total hormone H_0 or of dilution V_t/V_0 by means of the following equation which is a particular form of (I-10):

$$H_0 = F \sum_{i=1}^{2} \frac{P_{i0}}{K_1 + F} + F \sum_{i=3}^{5} \frac{P_{i0}}{K_i} + F(V_t/V_0) \qquad \text{(IB-1)}$$

After partial solving for F and introducing R for $\Sigma\, P_{i0}/K_i$ and L for V_t/V_0 one obtains:

$$F = H_0/\left(\frac{P_1}{K_1 + F} + \frac{P_2}{K_2 + F} + R + L\right) \qquad \text{(IB-2)}$$

From this relation, which may be symbolised by:

$$F = g(F) \qquad \text{(IB-3)}$$

The sequence

$$F_j = g(F_{j-1}) \qquad \text{(IB-4)}$$

which must converge, can be generated.
The calculation program consists of a routine for evaluating F_j from (IB-2) for $F = F_{j-1}$ which was the result of the preceding cycle and storage of the resulting F_j from which F_{j+1} will be calculated in the next, identical cycle. With each iteration step, consecutive estimates of F will come closer together. For generating the results of IA.1.2, IA.2.2 and 3 between 5 and 10 cycles were sufficient to obtain constancy of F up to the sixth significant figure. Keyboard operations and the use of storage facilities are shown in table IB.1.

Programming instructions:

Step No./Key	
	lrn
0	sto
1	0
2	+
3	rcl
4	1
5	=
6	1/x
7	*
8	rcl
9	3
10	+
11	rcl
12	4
13	:
14	(
15	rcl
16	0
17	+
18	rcl
19	2
20	)
21	+
22	rcl
23	5
24	=
25	:
26	rcl
27	6
28	=
29	1/x
30	R/S
31	Rst

Storage of variables:

Variable/memory No.	
F	0
K_1	1
K_2	2
P_1	3
P_2	4
R + L	5
H_0	6

Operating Instructions:

Key		Display
-	Rst	-
$F_{j=0}$	R/S	$F_{j=1}$
	R/S	$F_{j=2}$
	etc.	

Table I B.1
Programming and operating instructions for evaluating free hormone concentration as a function of total hormone and dilution on a Texas Instruments TI-51-III calculator.

Appendix IC

Mathematical details: The influence of dilution on F and F_0'.

In (I.4.) it was stated that F_0' is always a better estimate of F_0 than F, since $F < F_0' \leq F_0$.
In order to prove this inequality, first it will be demonstrated that $F < F_0$.
From (I-13a) and (I-13b) one can derive:

$$F/F_0 = \left(\Sigma \frac{P_{i0}}{K_i + F_0} + 1\right) / \left(\Sigma \frac{P_{i0}}{K_i + F} + (V_t/V_0)\right) \qquad \text{(IC-1)}$$

which can be rearranged to:

$$(V_t/V_0) = F_0/F + \Sigma \left(\frac{P_{i0}}{(F/F_0)K_i + F} - \frac{P_{i0}}{K_i + F}\right) \qquad \text{(IC-2)}$$

The expression between parentheses is positive when $F < F_0$ so that in that case $V_t/V_0 > F_0/F > 1$, since V_t/V_0 corresponds to dilution. On the other hand, if $F > F_0$, the expression between parentheses is negative, so that $V_t/V_0 < F_0/F < 1$, which corresponds with concentrating. Thus, two facts about the effects of dilution are demonstrated: $F < F_0$ and $V_t/V_0 > F_0/F$. The first inequality is used in the following derivation.
From (I-12c) one obtains for F_0:

$$F_0 = \frac{H_0}{\Sigma \frac{P_{i0}}{K_i + F_0} + 1} \qquad \text{(IC-3)}$$

Additionally, combination of (I-12c) and (I-14) gives for F_0':

$$F_0' = \frac{H_0}{\Sigma \frac{P_{10}}{K_1 + F} + 1} \qquad \text{(IC-4)}$$

And, since $F_0 > F$, also $F_0 \geq F_0'$. ($F_0' = F_0$ when $F_0 << K_1$). Comparison of (IC-4) with (I-12c) shows, since $V_t/V_0 > 1$, that also $F < F_0'$, so in conclusion:

$$F < F_0' \leq F_0$$

Chapter II

FREE HORMONE ASSAY BY SYMMETRIC DIALYSIS - THEORY.

II.1. Introduction.

One important conclusion to be drawn from the previous chapter is, that the changes of free hormone concentration induced in a sample by the application of equilibrium dialysis only in certain cases permit accurate assessment of the original free hormone concentration (ch.I.5. and ch.IV.2.).
In other words, often no adequate information about the original free hormone concentration can be obtained from the state at dialysis equilibrium.
In this chapter an alternative approach is introduced, in which a serum sample, to which tracer hormone is added, is dialysed against an identical sample (without tracer), so that a symmetric arrangement results. Besides, instead of the distribution of tracer hormone at equilibrium, the rate at which this equilibrium distribution is approached, is assessed as a measure of the free hormone fraction. Thus, since the process consists merely of redistribution of a tracer amount of radioactive labelled hormone after its addition to one compartment, the sample remains in its original state so that the dilution effect which is inherent in equilibrium dialysis, is absent. Both symmetric and conventional dialysis processes may be considered as particular cases of a general dialysis process which takes place in a dialysis system consisting of a membrane which is placed between two compartments of arbitrary size containing arbitrary concentrations of binding protein, and in which the approach towards equilibrium is monitored by means of added radioactive tracer hormone.
A mathematical description of the time-dependence of tracer distribution will be derived for this general model first (II.2.).
This approach has the advantage that, besides the mathematical principle of symmetric dialysis (II.3.), which is the most relevant for the present study, it yields additional information about three other modes of dialysis, including the conventional equilibrium

dialysis procedure (Appendix IIA).
The general model is based on a number of simplifying assumptions concerning constancy of the free hormone fraction and the rates of processes involved in transfer of tracer from one compartment to the other. Some of the assumptions are automatically fulfilled in the case of symmetric dialysis. Two assumptions, concerning rate-determining factors other than diffusion through the membrane, require closer consideration (II.4.).

II.2. A general model for the rate of dialysis processes.

The mathematical description of the time course of a general dialysis process as will be derived in this paragraph, will serve, as stated in the introduction to this chapter, not merely as a first step in the derivation of the mathematical principle of the symmetric dialysis method. Four different dialysis procedures - including symmetric dialysis - will be considered with regard to their possible suitability for obtaining some measure for the free hormone concentration by means of assessment of the rate at which equilibrium is approached. When also the relative ease, with which some of the assumptions underlying the general model are fulfilled, is taken into account, the symmetric dialysis procedure is by far best suited. The other three procedures are or may be more useful either for assays other than for free hormone or they must be applied under different conditions (equilibrium) and therefore these are discussed separately - also with help of the general model - in the appendix to this chapter.
When describing a general dialysis process the term dialysate and dialysand are less useful, since either or both compartments may, in the general case, containing binding protein.
Additionally, as the equivalence of dialysis and dilution will hold only in the particular case that one compartment does not contain binding protein and both compartments are of finite size (conventional equilibrium dialysis), the definition of the free hormone fraction as given in ch.I.5. is not useful any more. Instead, the free hormone fraction will be defined as the ratio of free to total hormone in a certain (homogeneous) compartment.

The following symbols are used:

c_1, c_2 : Concentration of radioactive labelled hormonal ligand in the first and second compartment.

c_0 : Concentration of tracer at time zero in the half-cell to which it is added at the start of the process.

f_1, f_2 : Free labelled (and unlabelled) hormone fractions in half-cells 1 and 2.

V_1, V_2 : Volumes of solution in compartments 1 and 2.

A : Membrane area.

h : Membrane thickness.

With help of these symbols the four procedures that will be discussed as particular cases of the general model are readily characterised (see also fig.II.1):

1). Equilibrium dialysis, "conventional type". At t = 0, the first compartment contains tracer and binding protein, the second only solvent or buffer so that $f_2 = 1$. At equilibrium the first compartment contains bound + free tracer, in the second compartment only free tracer is present. The free fraction is calculated as the ratio of the radioactivity in compartment 2 to that in the first compartment (c_2/c_1).

2). Equilibrium dialysis "reverse". At t = 0 the first compartment contains tracer, the second the binding proteins so that $f_1 = 1$. The equilibrium state is equal to that of process 1), with first and second compartments exchanged. The direction, however, from which equilibrium is approached is the opposite of that in the conventional case.

3). Dialysis against infinite or pseudo-infinite volume. The first compartment contains at t = 0 both tracer and binding protein. The second compartment is either of infinite volume, ($V_2 = \infty$), or it contains an extremely strong binding or adsorbing agent ($f_2 = 0$), so that in either case the free hormone concentration is virtually zero. The process proceeds until all tracer has disappeared (infinite volume) or has migrated into the second compartment (pseudo-infinite volume).

4). "Symmetric dialysis". First and second compartments contain identical solutions of binding proteins: $f_1 = f_2 = f$. Tracer is

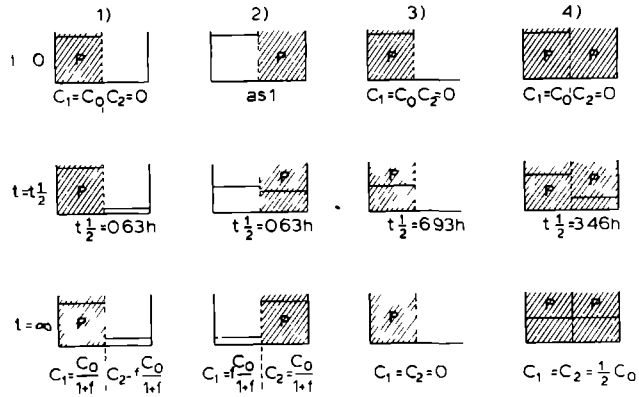


Fig. II.1.

Four different procedures for performing dialysis (1)-4), see text).
The tracer levels c_1 and c_2 in each compartment are represented by horizontal lines at the beginning, halfway and at equilibrium for the case that the free hormone fraction f is 0.1. The symbol P denotes the presence of binding protein.

added initially to the first compartment and at equilibrium both compartments contain the same concentration of tracer.

As the general model should comprise all four types of procedures the following assumptions must be made in order to keep the model workable:

1. Hormone is transported through the membrane only in the unbound form by means of diffusion, so that Fick's laws are obeyed.

2. *Labelled and unlabelled hormone are, within each compartment,* equally distributed over free and protein bound moieties.

3. The concentration of unbound tracer hormone immediately at the membrane surface is both identical to the free concentration in the bulk of the solution and in equilibrium with the concentration of bound tracer. This implies that the concentration drop of free tracer over the membrane is not influenced by:
a. the rate at which tracer is transported towards and from the membrane by convection, i.e. agitation efficiency of the contents of each half-cell must be infinite.
b. the association or dissociation rate of tracer-protein complexes.

4. *Constancy of* the free hormone fraction in each compartment. The free hormone fraction, when defined per half-cell as above, may be affected by two processes, which are assumed not to take place or to have no effect: changes in total hormone concentration as a consequence of redistribution of either or both labelled and unlabelled hormone and dilution by fluid shift which also would affect the relative magnitudes of V_1 and V_2.
The latter assumption is almost redundant in the case of symmetric dialysis, since fluid shift due to osmotic pressure cannot occur between identical solutions, and redistribution only of tracer hormone, which should therefore be added in real tracer amounts, takes place The same assumption is rather restrictive to the applicability of the general model to conventional equilibrium dialysis and even more to the type 3) procedure, since there all hormone disappears from the first compartment during the process. It requires, in addition to the absence of fluid shift that changes in free hormone concentration during dialysis are very small relative to

the dissociation constants involved (see eq.I-11).
Also the first assumption - the statement that diffusion is the sole mode of transport of the hormone through the membrane - is clearly acceptable in the symmetric dialysis case as transport by convection supposes (osmotic) pressure differences which can not exist between identical solutions.
With symbols and conditions defined, the general model is derived as follows.
Initially, tracer is added to the first compartment, so at $t = 0$, $c_1 = c_0$ and $c_2 = 0$. In general, at any point in time:

$$c_0 = \frac{V_1 c_1 + V_2 c_2}{V_1} \qquad \text{(II-1)}$$

Fick's first law states that the flow by diffusion of material per unit time through unit cross-section is proportional to the concentration gradient of the diffusing material. So the total tracer flow S in mass per unit time through the membrane from one compartment to the other is proportional to the concentration gradient of diffusible (= free) tracer over the membrane and to the membrane area A.

$$S = A\, D_m \frac{f_1 c_1 - f_2 c_2}{h} \qquad \text{(II-2)}$$

The proportionality factor D_m ($m^2.sec^{-1}$) includes the "ordinary" diffusion coefficient in the solvent in the membrane pores and a factor which relates the summed cross-sections of the pores to the external membrane area. It may also include an interaction coefficient. For convenience, as membrane thickness h is also a property of the membrane it will be incorporated in the "membrane diffusion coefficient" D ($D = D_m/h$). By this operation the dimensions of D become that of a rate instead of area per unit time, as applies for the "ordinary" diffusion coefficient.
The change of concentration c_2 with time, as tracer diffuses into compartment 2 is equal to the flow S divided by the compartment

volume:

$$\frac{dc_2}{dt} = \frac{S}{V_2} \qquad \text{(II-3)}$$

Combined with (II-2):

$$\frac{dc_2}{dt} = \frac{AD}{V_2}(f_1c_1 - f_2c_2) \qquad \text{(II-4)}$$

From (II-1) follows:

$$c_1 = c_0 - (V_2/V_1)c_2 \qquad \text{(II-5)}$$

which is substituted in (II-4):

$$\frac{dc_2}{dt} = \frac{AD}{V_2}(f_1c_0 - c_2(f_1\frac{V_2}{V_1} + f_2)) \qquad \text{(II-6)}$$

of which the general solution is (using the substitution rule for integrating):

$$\ln(f_1c_0 - c_2(f_1\frac{V_2}{V_1} + f_2)) = -(\frac{f_1}{V_1} + \frac{f_2}{V_2})\,A\,D\,t + C \qquad \text{(II-7)}$$

The constant C is found with help of the boundary condition $c_2 = 0$ at $t = 0$:

$$C = \ln f_1c_0 \qquad \text{(II-8)}$$

Substitution in eq.(II-7) and rearrangement gives:

$$\ln(1 - \frac{c_2}{c_0}(\frac{V_2}{V_1} + \frac{f_2}{f_1})) = -(\frac{f_1}{V_1} + \frac{f_2}{V_2})\,A\,D\,t \qquad \text{(II-9)}$$

which describes the time course of any dialysis process fulfilling the conditions given in II.2.
Alternatively it may be written after partial substitution of eq.(II-1) as:

$$\ln \frac{f_1c_1 - f_2c_2}{f_1c_0} = - \left(\frac{f_1}{V_1} + \frac{f_2}{V_2}\right) A\ D\ t \qquad \text{(II-10)}$$

from which it appears that the first member represents the natural logarithm of the ratio of the concentration drop of free tracer over the membrane at time t: $f_1c_1 - f_2c_2 = \Delta fc_{(t)}$ to that at $t = 0$: $f_1c_0 = \Delta fc_{(0)}$. This ratio, which is a measure of how far the process is away from equilibrium, will be denoted by Q_t, and it will range from 1 to 0 for $t = 0 \ldots\ldots \infty$, so that $\ln Q_t$ ranges from 0 to $-\infty$.
Equilibrium is reached when $\Delta fc_{(t)} = 0$ so that also $Q_t = 0$.
So:

$$\frac{f_1c_1 - f_2c_2}{f_1c_0} = \frac{\Delta fc_{(t)}}{\Delta fc_{(0)}} = Q_t \qquad \text{(II-11)}$$

and:

$$\ln Q_t = - \left(\frac{f_1}{V_1} + \frac{f_2}{V_2}\right) A\ D\ t \qquad \text{(II-12)}$$

The exponential relation between Q_t and time makes it possible to define a time-independent parameter of the process.
The dialysis rate* U thus will be defined as the change with time of $\ln Q_t$ so that it is an indicator of the rate at which equilibrium is approached. Combination of the definition $U = -(1/t)\ln Q_t$ with eq.(II-12) shows that U is independent of dialysis time:

* Strictly spoken, the term "fractional dialysis rate" would be more appropriate since it is defined on the fraction of equilibrium attained. Since no misunderstanding is expected the term dialysis rate is used for shortness.

$$U = -(1/t)\ \ln Q_t = -(1/t)\ \ln \frac{f_1c_1 - f_2c_2}{f_1c_0} = \left(\frac{f_1}{V_1} + \frac{f_2}{V_2}\right) A\ D \qquad (II\text{-}13)$$

In this relation, the variables c_1, c_2, c_0 eq.(II-1) and t are measurable. This is not sufficient for assessing U because of the presence of f_1 and f_2, which are unknowns, under the ln sign. In fact, U can be assessed only in those particular cases where both f's disappear from the expression, as will be the case when $f_1 = f_2$ (symmetric dialysis - type 4) or $f_2c_2 = 0$ (infinite or pseudo-infinite volume dialysis - type 3), fig. II.1. For the equilibrium dialysis procedures either f_1 or f_2 is equal to unity but the other f remains under the ln sign, so that U can not be assessed. This eliminates procedures 1) and 2) as possible approaches for the determination of the free hormone fraction f from dialysis rate measurements. Instead, these methods yield at equilibrium a straightforward estimate of f (as c_2/c_1 or c_1/c_2). Eq.(II-13) is still very useful in these cases in predicting the time course and the time required for attainment of equilibrium for these and other procedures in which the tracer distribution at equilibrium has the greater significance (Appendix IIA).

In conclusion it may be stated that symmetric dialysis (procedure 4) as a kinetic method for determining the free hormone fraction from dialysis rate measurements is the better approach of all four procedures viewed as rate methods. Partly this is obvious, since procedures 1) and 2) even do not permit assessment of their dialysis rate U.

On the other hand, symmetric dialysis is also preferred to the infinite or pseudo-infinite mode of dialysis (3)) because the requirements for the theoretical validity (particularly assumptions 1 and 4) of the derived relation between free hormone fraction and dialysis rate are much easier fulfilled.

II.3. The principle of symmetric dialysis.

In the previous paragraph it was shown that for a symmetric dialysis process in which the solutions of binding proteins on both

sides of the membrane are identical so that $f_1 = f_2 = f$ and $V_1 = V_2 = V$, the dialysis rate U is directly assessible from measurements of c_1 and c_2 at a certain time t, since in that case (II-13) reduces to:

$$U = - (1/t) \ln \frac{c_1 - c_2}{c_0} = 2fAD/V \qquad \text{(II-14)}$$

Now f has disappeared from the expression under the ln sign which means in terms of the definition of Q_t (eq.II-11) that the ratio of free tracer concentration differences has become equal to the ratio of concentration differences for total tracer. So Q_t and thereby U have become readily assessible from the distribution of total tracer at a certain point in time. In this instance Q_t will be replaced by the symbol I which stands for isotope distribution.

Practical assay of the free hormone fraction through measurement of the dialysis rate is based on eq.(II-14). Tracer is added at t = 0 in compartment 1 and after dialysing for a certain time t following tracer addition, the process is ended and c_1 and c_2 are determined (and $c_0 = c_1 + c_2$). From these values tracer distribution $I = (c_1 - c_2)/c_0$, its natural logarithm ln I and the dialysis rate $U = -(1/t)\ln I$ are calculated.

Since U is independent of time, it can be estimated from c_1 and c_2 values at any point in time. Preferably a dialysis period is chosen in which c_2 has become large enough to permit precise measurement of $(c_1 - c_2)/c_0$. This is perhaps the most important advantage of symmetric dialysis over equilibrium dialysis because in the latter case one is confined to assay of c_2/c_1 (=f) at equilibrium so that assay precision always depends directly on f. In contrast, in symmetric dialysis almost always the same precision, regardless of f, is attainable by proper choice of the dialysis time. The possibility of increasing c_2 by prolonging the dialysis time further results in a much lower susceptibility towards interference from tracer impurities than encountered in equilibrium dialysis, as will be shown in chapter V together with a more extensive treat-

ment of the precision of symmetric dialysis. In order to obtain f, the product 2AD/V must be known yet. This product consists of three constants characteristic of the dialysis cell equipment used and therefore will be denoted as "cell permeability constant". This constant can be obtained by applying the above procedure leading to U, to a reference solution with known f.
Problems connected with such standardisation procedures are dealt with in V.3.3.

An impression of the actual dialysis rates to be encountered can be obtained when deriving the half-life of the process from the general expression (II-13):

$$t_{\frac{1}{2}} = \ln 2 \; (A \; D \; (f_1/V_1 + f_2/V_2))^{-1} \qquad \text{(II-15)}$$

which was obtained by substituting $Q_t = 0.5$ in eq.(II-11). This means that $t_{\frac{1}{2}} = \ln 2 (2ADf/V)^{-1}$ for symmetric dialysis. When for the product AD/V a value of 1 h^{-1}, being of the order of magnitude as found in practice, is taken, one finds for f = 0.01 a $t_{\frac{1}{2}}$ of 34.6 h. The $t_{\frac{1}{2}}$ is inversely proportional to f. When this value is compared with the half-life of a conventional equilibrium dialysis process (0.69 h, same values for AD/V and f) it is clear that the symmetric process is remarkably slower. This may be intuitively understood by realising that with only one hundredth of total tracer in free (diffusible) form, the symmetric process ends eventually with half the amount of total tracer transported to the other compartment, whereas in equilibrium dialysis only 1% has to be transferred.
In practice this difference in the rates of approach to equilibrium generally does not lead to large differences in time needed for the assay, since equilibrium dialysis is carried on until equilibrium is attained, whereas the symmetric dialysis process is ended very much earlier. As will be shown in ch.V.4., better precision in the measurement of I is obtained when it is somewhere between 0.2 and 0.75. With the above values of AD/V and f, dialysis indeed on the average will be carried on for about one half-life. In the free T4 assay, AD/V ranges from 2 - 2.5, depending on the membrane, and values of f generally range from 0.005 to 0.015. When the

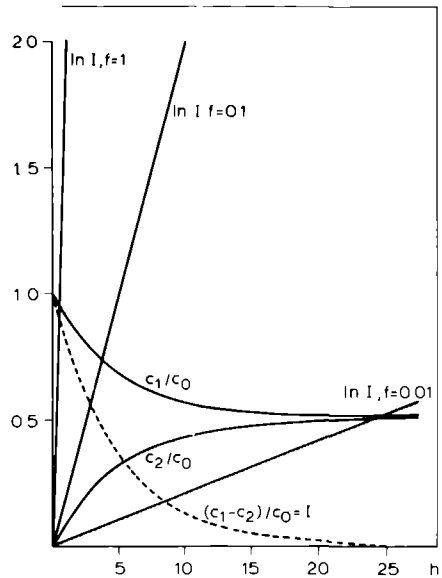


Fig. II.2.

Approach to equilibrium of a symmetric dialysis process for AD/V = 1 and f = 1. Shown are the changes with time of tracer concentrations c_1 and c_2 and their difference, relative to the initial tracer concentration c_0. In addition, ln vs t curves are given for f = 1, f = 0.1, and f = 0.01. The slopes of these curves represent the corresponding dialysis rates.

process is ended after 18 h, values of -ln I are between 0.35 and 1.35, while I ranges from 0.25 to 0.7. The time course of a symmetric dialysis process is shown in fig.II.2.

II.4. The rate-limiting step in symmetric dialysis.

In the previous paragraph the principle of determination of free hormone fractions from measurements of the dialysis rate in a symmetric arrangement was introduced. Some striking advantages this method would offer as compared with equilibrium dialysis could be discerned, but it was also pointed out that the mathematical model on which the method relies and from which it derives its theoretical validity involves four essential assumptions, two of which are exclusively relevant to rate methods. As the extent to which these are fulfilled is expected to have a large bearing on the accuracy of the symmetric dialysis method these assumptions require closer consideration. For convenience they are repeated here, formulated in a slightly different way:

A. In spite of the fact that continuously tracer is transferred from one compartment to the other, tracer hormone concentration is the same near the membrane and in the bulk of the solution. Its value does not depend on the location within the half-cell. Therefore the contents of each half-cell must be agitated with infinite efficiency.
B. The ratio of free to total tracer (free tracer fraction = free hormone fraction) retains its original, before dialysis value. This implies that association and dissociation rates are fast enough to maintain equilibrium between free and bound tracer, although continually free tracer migrates through the membrane.

Although different processes are involved, no other process besides membrane diffusion determines the overall rate of tracer transfer when these assumptions hold. This is reflected in the direct proportionality between U and f according to eq.(II-14).
It appeared however from experimentation that this linear relation between U and f not always holds. Therefore it was found necessary to investigate whether and on what conditions these assumptions

are justified. When doing so, the first step is to derive for a symmetric arrangement a general expression that relates the dialysis rate to the drop of free tracer concentration over the membrane but that does not depend on either assumption A or B (II.4.1). Subsequently it is considered what processes - already referred to by assumptions A and B - are involved in transport of the tracer molecules from one compartment to the other and thereby may affect the free tracer concentration drop over the membrane. The contribution of tracer transport in solution to the concentration drop was laid down in a model based on Nernst's film diffusion concept (II.4.2), whereas the possible influence of dissociation and association rate of the tracer-protein complexes also could be formulated mathematically (II.4.3).

II.4.1. Free tracer concentration drop over the membrane in relation to the dialysis rate: Derivation without simplifying assumptions.

Since the discussion in this and the next paragraphs will be confined to the symmetric type of dialysis, the general expression for the dialysis rate, which takes into account additional rate-controlling factors, will be derived directly for the conditions of symmetric dialysis, i.e. $f_1 = f_2 = f$ and $V_1 = V_2 = V$.
The flow S of tracer through the membrane at a certain point in time is proportional to the difference $\Delta\hat{F} = \hat{F}_1 - \hat{F}_2$ between the concentrations of free (diffusible in the membrane) tracer in the solutions that are in direct contact with the two membrane surfaces:

$$S = \frac{AD_m}{h} \Delta\hat{F} \qquad \text{(II-16)}$$

where D_m denotes the diffusion coefficient in the membrane and $\hat{F}$ is introduced as the concentration of free tracer hormone. The changes in the concentrations c_1 and c_2 of total tracer in compartments 1 and 2 in a time dt are:

$$dc_1 = \frac{AD_m}{V_h} \hat{\Delta F}\, dt \qquad \text{(II-17a)}$$

$$dc_2 = \frac{AD_m}{V_h} \hat{\Delta F}\, dt \qquad \text{(II-17b)}$$

Hence the rate of change of the concentration difference of total tracer $\Delta c = c_1 - c_2$ is given by:

$$\frac{d\Delta c}{dt} = -2 \frac{AD_m}{Vh} \hat{\Delta F} \qquad \text{(II-18)}$$

which can be written also as:

$$\frac{d\Delta c}{dt} = -2 \frac{AD_m}{Vh} (\hat{\Delta F}/\Delta c)\Delta c \qquad \text{(II-19)}$$

Now, if $\hat{\Delta F}/\Delta c$ is a constant and since $\Delta c = c_0$ at $t = 0$, eq.(II-19) can be integrated to:

$$\ln \frac{\Delta c}{c_0} = -2 \frac{AD_m}{Vh} (\hat{\Delta F}/\Delta c)\, t \qquad \text{(II-20a)}$$

Division by t gives the dialysis rate U:

$$U = 2 \frac{AD_m}{Vh} (\hat{\Delta F}/\Delta c) \qquad \text{(II-20b)}$$

of which (II-14) is a particular case for $\hat{\Delta F}/\Delta c = f$.
About the $\hat{\Delta F}/\Delta c$ ratio the following must be stressed:
In the above derivation $\hat{\Delta F}$ was defined as the difference of free tracer concentrations at the membrane-solution interfaces on each side of the membrane, thus representing the "driving force" of the membrane diffusion process. It leaves open the possibility that these concentrations are not equal to those found in other locations

within the same compartments and that they do not correspond to the equilibrium of tracer and binding proteins. In contrast, Δc refers to the net difference in total tracer content of the half-cells. It is the quantity that is actually measured and it does in no way reflect non-uniformity of concentrations within the system or non-equilibrium conditions.

Furthermore, $\hat{\Delta F}/\Delta c$ is assumed to be independent of time so that integration from (II-19) to give (II-20) can be carried out. Should this not be the case in practice, a non-linear relation between $\ln (\Delta c/c_0) = \ln I$ and dialysis time would be observed. Constancy of dialysis rate U with time must however be considered as a fundamental prerequisite for performing symmetric dialysis. This is the reason why the effects of transport in solution and forward and backward reaction on $\hat{\Delta F}/\Delta c$ only are studied for the case that this ratio is constant. A particular tracer hormone molecule must, in order to penetrate the membrane, first be transported towards the membrane by convection and (for a more or less significant proportion) by diffusion and it also must dissociate from its binding proteins. On the opposite side of the membrane the molecules arriving at the surface by membrane diffusion must be carried away into the bulk solution and become associated again with the binding proteins. These processes, transport in solution, transport in membrane, association and dissociation all may affect to some extent the concentration drop of free tracer over the membrane, and thereby the dialysis rate (II-20b). When transport of hormone towards the membrane is slow, the balance between supply of free hormone at the membrane surface and its removal by membrane diffusion may require a lower concentration difference of free tracer over the membrane than at higher (in solution) transport rates. A lowered concentration difference will also result from a low rate of supply of free tracer hormone at the membrane surface as a consequence of dissociation of hormone-protein complexes. On the opposite side of the membrane, a further decrease of the free concentration difference may result from accumulation of free tracer which has just passed the membrane, either because it associates slowly with its binding proteins or because it is only slowly carried by convection.

II.4.2. Transport in solution as a rate-controlling step: The film-diffusion concept.

Assumption A states that hormone is transported towards and from the membrane by convection (stirring) and that this process is very rapid as compared with diffusion through the membrane, thus resulting in equal concentrations of all species at any location in the dialysis half-cell. This is a description of an unrealistic situation, for the velocity of convection inevitably falls in the neighbourhood of the membrane and it even becomes zero at the membrane/solution interface. Under these circumstances transport of tracer hormone towards and from the membrane must take place by means of a different mechanism, viz. diffusion. At some distance of the membrane, diffusion forms only a minor contribution to overall transport but its importance increases nearer to the membrane surface until at a very short distance it has become virtually the sole mode of transport. A working model for this situation was formulated by Nernst in the film or diffusion layer concept. In this concept, a zone of defined thickness adjacent to the membrane where diffusion is the only mode of transport of a solute and which is separated from the completely agitated solution by a sharp boundary, replaces a wider region of continuously decreasing convection flow rate and increasing relative importance of diffusion (fig.II.3).
At moderate tracer fluxes*, the diffusion rate in the layer may be sufficient to maintain an even concentration in the layer, which is equal to that in the bulk solution. At higher fluxes, temporarily more tracer may disappear into the membrane than is supplied by diffusion and a concentration gradient will develop in order to establish a new balance. As soon as this happens, diffusion through the membrane is not the only controlling factor of the overall dialysis rate, but the process is now also partially under film diffusion control.

* Flux: Amount of substance crossing unit area per unit time ($mol.m^{-2}.sec^{-1}$).

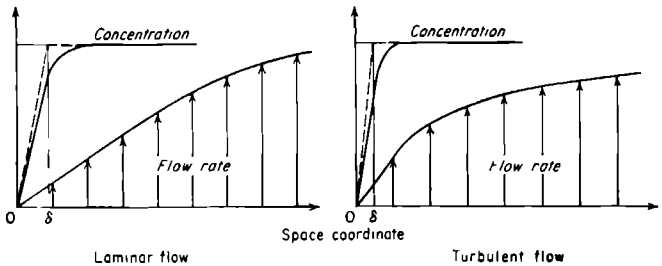


Fig. II.3.

The Nernst diffusion layer. The diagrams show the actual and the idealized concentration profiles (solid and broken lines, respectively) of a dissolved species which reacts instantaneously at the solid surface. The idealized profile is the tangent of the actual profile at the surface. In addition, the actual flow rates of the solutions are shown (Helfferich 1962).

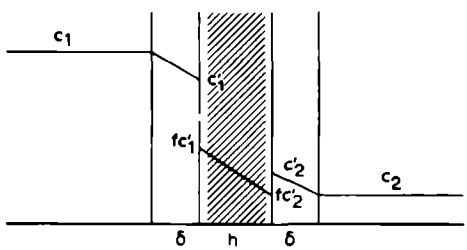


Fig. II.4.

Application of the film diffusion concept to symmetric dialysis. Concentration profiles in solution, diffusion layers and membrane.

Fig.II.3 shows actual and idealized concentration profiles in the neighbourhood of the membrane for the case of an infinite speed of membrane diffusion, causing zero concentration of solute at the membrane/solution interface. It also shows that the type of agitation (laminar vs.turbulent flow) plays an important role with regard to film thickness.

Now it must be considered what consequences for the symmetric dialysis method are to be expected when film diffusion partially controls the dialysis rate. The crucial point is that whereas the membrane is only permeable to free hormone, the diffusion layer allows passage of both free and bound hormone. The most important consequence of both processes being rate-controlling is that the proportionality between the free hormone fraction and the dialysis rate is lost. This can be proven by the derivation of an expression for $\Delta\hat{F}/\Delta c$ which takes into account the influence of the diffusion layer. Therefore the model used hitherto is extended to include two layers of solvent of thickness δ adjacent to the membrane in which tracer hormone (both free and bound) is transported solely by diffusion, with coefficient D_ℓ. The tracer concentrations in the bulk solution of both compartments are c_1 and c_2. Since partial film diffusion control is assumed, concentration gradients in the films will have developed such that in the solutions immediately at the membrane surface the tracer concentrations are c_1' and c_2', so, that $c_1 > c_1'$ and $c_2' > c_2$ (fig.II.4).

It is assumed that the tracer flux is constant along the horizontal axis. The latter implies that the complete concentration profile for the diffusion layer-membrane system consists only of linear segments. As diffusion is the only mode of transport and the tracer flux J is equal for films and membrane, the following expression may be written:

$$J = \frac{c_1 - c_1'}{\delta} D_\ell = \frac{f\,(c_1' - c_2')}{h} D_m = \frac{c_2' - c_2}{\delta} D_\ell \qquad \text{(II-21)}$$

f is used instead of $\Delta\hat{F}/\Delta c'$ because it is assumed that the conditions for assumption B are fulfilled. Now, since

$$\hat{\Delta F} = f\ (c_1' - c_2')$$

and

$$\Delta c = c_1 - c_2$$

(which indicates that in general $\hat{\Delta F}/\Delta c \neq f$)
from (II-21) one can derive:

$$(\hat{\Delta F}/\Delta c) = f/(1 + \frac{2fD_m\delta}{D_\ell h}) \qquad \text{(II-22)}$$

From (II-22) a clear picture can be obtained of which factors are favourable to film diffusion control and how the opposite is promoted, namely $\hat{\Delta F}/\Delta c$ reaching its ideal value f under complete membrane diffusion control. With partial film diffusion control (II-22) demonstrates that no linear relation exists any more between $\hat{\Delta F}/\Delta c$ and f. Therefore the dialysis rate also is no linear function of f (see (II-20b)). Factors counteracting film diffusion control are the use of dense (D_m <), thick (h>) membranes, efficient agitation ans low viscosity of the solutions (δ<) and, which is very important, low values of f.
Two additional useful expressions for U follow from (II-20) and (II-22):

$$U = \frac{2AD_m}{hV}\,\frac{f}{\frac{2fD_m\delta}{D_\ell h} + 1} = \frac{2Af}{V(\frac{2\delta f}{D_\ell} + \frac{h}{D_m})} \qquad \text{(II-23a, b)}$$

Also here the influence of factors promoting or counteracting film diffusion control are discernible. Especially in eq.(II-23b) the appearance of the term $2\delta f/D_\ell$ demonstrates the lowering effect of film diffusion on the dialysis rate. When this term becomes negligible, eqs.(II-23) reduce to (II-14).
The foregoing may have important consequences for the practical

application of the symmetric dialysis method. If the values of D_m, D_ℓ and δ are such, that membrane diffusion prevails, the simple model of II.3. applies and there is obviously no problem. However, it is possible that only at low values of f the rate is fully controlled by membrane diffusion. The range of these values should then be determined experimentally by assessing the influence of stirring speed on the dialysis rate (varying δ). Observation of the absence of any influence does however not completely exclude the possibility that film diffusion is involved. Alternatively one may observe the ratio of dialysis rates obtained with different membranes for increasing values of f. As long as this ratio remains constant, very probably only membrane diffusion is rate-limiting. Ideally, the cell permeability constant, which must be known in order to calculate f from the observed dialysis rate U for an unknown sample is, according to eq.(II-14) equal to U/f observed for a reference solution with known f. This approach is valid when this ratio is independent of f. Partial film diffusion control cuases the cell permeability constant U/f to be dependent on the f of the reference solution used for its determination (see eq.(II-23)). Therefore it is necessary either to restrict measurements of cell permeability constants and unknown f's to the range of f where only membrane diffusion is rate-limiting or to construct U vs. f standard curves by means of reference solutions, on which unknowns can be read (see III.1.3 and V.2.2).

With regard to the film diffusion coefficient D_ℓ some further refinements are necessary. It is composed of the various diffusion coefficients of the binding proteins (D_1) in addition to that of the unbound hormone (D_f). The contribution of each diffusion coefficient to D_ℓ evidently must be related to $b_1 = B_1/H$ i.e. the fraction of total hormone that is transported by the corresponding binding protein.
So for D_ℓ one should write:

$$D_\ell = \Sigma b_1 D_1 + f\, D_f \qquad \text{(II-24)}$$

Since D_1 will be generally much smaller than D_f, the film diffusion

coefficient will increase when binding is decreased e.g. by dilution. At the same time viscosity may decrease so that all diffusion coefficients rise. Both effects counteract the direct influence of the free hormone fraction on the contribution of film diffusion (II-23b).
Experimental evidence for the efficacy of the film diffusion concept to explain departures from ideal behaviour according to (II-14) is given in ch.V.3 and the appendix to ch.V.

II.4.3. Association or dissociation reactions as rate controlling steps.

The purpose is now to express the ratio $\Delta\hat{F}/\Delta c$ in terms of the rates of dissociation of the tracer binding protein complexes. Then again will be studied under what conditions this ratio attains its ideal value f, and how the dialysis rate will depend on reaction rates in the general case. This time it is assumed that only assumption A holds: concentrations of free and total hormone at the membrane surface are equal to those in the bulk of the solution.
An expression for $\Delta\hat{F}/\Delta c$ can be obtained from the differential equations describing the change in free tracer concentration in each compartment as resulting from dissociation, association and transfer into or out of the compartment according to the schematic representation of fig.II.5:

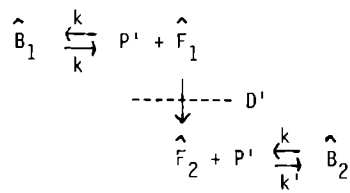


Fig.II.5.

Reactions involved in transport of tracer from one compartment to the other.

Symbols used:

$\hat{B}_1$, $\hat{B}_2$: Concentrations of protein bound tracer in first and second compartment.

$\hat{F}_1$, $\hat{F}_2$: Concentrations of free tracer in 1st and 2nd compartment.

P' : Concentrations of all unoccupied binding sites present (assumed to be equal for both compartments).

k' : Mean rate constant for all association reactions.

k : " " " " " dissociation reactions.

D' : Cell permeability constant = rate constant for the transfer reaction through the membrane (AD_m/hV, eqs.(II-17)).

The net rates of change of $\hat{F}_1$ and $\hat{F}_2$ are

$$\frac{d\hat{F}_1}{dt} = k\,\hat{B}_1 - k'\,P'\hat{F}_1 - D'(\hat{F}_1 - \hat{F}_2) \qquad \text{(II-25a)}$$

and

$$\frac{d\hat{F}_2}{dt} = k\,\hat{B}_2 - k'\,P'\hat{F}_2 + D'(\hat{F}_1 - \hat{F}_2) \qquad \text{(II-25b)}$$

The difference between (II-25a) and (II-25b) gives:

$$\frac{d\Delta\hat{F}}{dt} = k(\hat{B}_1 - \hat{B}_2) - k'P'\Delta\hat{F} - 2\,D'\Delta\hat{F} \qquad \text{(II-26)}$$

Substituting $c_1 = \hat{B}_1 + \hat{F}_1$ and $c_2 = \hat{B}_2 + \hat{F}_2$ results in:

$$\frac{d\Delta\hat{F}}{dt} = k(c_1 - c_2) - k(\hat{F}_1 - \hat{F}_2) - k'P'\Delta\hat{F} - 2\,D'\Delta\hat{F} \qquad \text{(II-27a)}$$

which simplifies to:

$$\frac{d\Delta\hat{F}}{dt} = k\Delta c - (k + k'P' + 2\,D')\,\Delta\hat{F} \qquad \text{(II-27b)}$$

Because $\Delta\hat{F}/\Delta c$ is assumed to be constant, the first member can also be written as $(\Delta\hat{F}/\Delta c)d\Delta c/dt$, while eq.(II-18), since $AD_m/hV = D'$, gives an expression for $d\Delta c/dt$ in terms of D', which may be inserted in (II-27b). Division by Δc and rearrangement gives:

$$D'(\Delta\hat{F}/\Delta c)^2 - (k + k'P' + 2\,D')(\Delta\hat{F}/\Delta c) + k = 0 \qquad \text{(II-28)}$$

From (II-28) conditions for k, k', P' and D' can be found such that $\Delta\hat{F}/\Delta c$ is equal to f:

Suppose $k >> 2D'$, then also $k >> D'(\Delta\hat{F}/\Delta c)^2$ and one obtains:

$$-(k + k'P')(\Delta\hat{F}/\Delta c) + k = 0$$

and since $f = k/(k'P' + k)$ (from the law of mass action):

$$(\Delta\hat{F}/\Delta c) = f \qquad \text{(II-30)}$$

The condition $k >> D'$ is not the only one leading to complete diffusion control:

Assume that now k is of the same order of magnitude as D' but that f is very small so that, since $f = k/(k'P' + k)$:

$$k'P' + k >> k$$

which combines with $k \approx D'$ to:

$$k'P' + k >> D'$$

Substitution in (II-28) yields again (II-29).

This result can be explained by the fact that the amount of free tracer generated per unit time is equal to the product of the dissociation rate constant and the concentration of bound tracer. So, if the rate constant is low, even as low as the rate constant for diffusion, it is still very well possible that a high concentration of bound tracer provides an adequate supply of free tracer to be transported through the membrane. Therefore it may be expected that there will be instances where the dissociation rate does not influence the dialysis rate at a certain concentration of binding proteins, but upon further dilution the concentration

of bound tracer becomes too low to have a sufficient supply of free tracer generated per unit time from it.
The following may give an impression of the orders of magnitude involved. The dissociation half-time at 37°C of T4 from serum is 20 sec. (Hillier, 1975) from which the dissociation rate constant k is calculated by means of $k = (-\ln 0.5)/t_{½}$ as $k = 0.0347\ sec^{-1}$. Let in a normal serum f_0 (T4) be 0.016% , then at a 1 : 50 dilution as applied for routine assay, f = 0.008. Since $f = k/(k + k'P')$ it follows that $k + k'P' = 4.375\ sec^{-1}$. For the combination: T4 - 1 ml cells - Visking membranes the cell permeability constant $D' = AD_m/hV$ is $2.25\ h^{-1} = 0.000625\ sec^{-1}$. Clearly this, also when multiplied by 2, can be neglected when compared with the value found for $k + k'P'$, so that again (II-29) emerges from (II-28). It is also possible to find out how slow a dissociation reaction may be just to prevent it from influencing the dialysis rate. In order to find such a condition it will be required that $k + k'P' \geq 100.2D'$ i.e. $0.125\ sec^{-1}$ so that $k \geq 0.125f$. From this condition one can readily calculate that at f = 0.1, k may be as low as $0.0125\ sec^{-1}$ which corresponds to a half-life of 55 sec. At f = 0.01 the $t_{½}$ might be as long as 9 minutes.

For completeness it will be shown when the dialysis rate is fully determined by the dissociation rate of the tracer binding protein complexes:
The dialysis rate U can be introduced using (II-20b) ($U = 2D'(\Delta\hat{F}/\Delta c)$):

$$U^2 - (k + k'P' + 2\ D')\ U + 2\ k\ D' = 0 \qquad \text{(II-31)}$$

which can be rearranged to:

$$U^2/D' - (k + k'P')U/D' - 2\ U + 2\ k = 0 \qquad \text{(II-32)}$$

from which it is easily seen that U approaches K for very high values of D'. In that case the dissociation of the hormone from its binding proteins is rate-limiting.

Serum to serum dialysis was applied for the first time by Christensen (1959). However, the method described by this author differs from the present one in a number of respects, as appears from a citation from the article: "The product of the above two values (the PBI and the percentage of the total amount of radiothyroxine added that has passed the membrane in 24 hrs. loc. cit.) is an expression of the concentration of free thyroxine in arbitrary units. An absolute value cannot be obtained by the proposed method". Indeed measurements were confined to the very first phase of the process (dialysis of undiluted serum-T4 tracer added) where only a minute proportion of tracer has migrated to the other compartment, so that in terms of the present study c_1 still virtually equals c_0, and c_2 is still negligible as compared with c_0. Since in addition $f_1 = f_2 = f$, $V_1 = V_2 = V$ and $D_m/h = D$, eq.(II-2) reduces to:

$$S = ADfc_0 \qquad \text{(II-33)}$$

eq.(II-3) then becomes:

$$dc_2/dt = fc_0AD/V \qquad \text{(II-34)}$$

which can be readily integrated. A boundary condition is $c_2 = 0$ at $t = 0$. The following result is obtained:

$$c_2/c_0 = ftAD/V \qquad \text{(II-35)}$$

The ratio c_2/c_0, always determined after the same dialysis period (24 h) was used by Christensen as the measurement parameter proportional to the free hormone (T4) fraction, since no attempt was made to measure AD/V.

Measurements in the initial phase with only a very small amount of tracer transferred is unfavourable to assay precision and also makes the method susceptible towards the effect of tracer impurities, although the latter was counteracted for a large part by means of a precipitation and washing step. In contrast, the symmetric dialysis method, as developed in the present study, is based on a

mathematical analysis of the time course of tracer distribution during the complete dialysis process. Thus it is possible to carry out the measurements almost at any point in time. In fact it is possible to choose the dialysis time in such a way that always the same measurement precision is obtained, irrespective of the value of the free hormone fraction (II.3 and also V.4).

For the derivation of the mathematical model of symmetric dialysis first the time course of a "general" dialysis process in which different concentrations of binding proteins are present in both compartments, was analysed. This was done or order to examine whether some of the known types of dialysis arrangements, which all may be considered as particular cases of the general case, are suitable for use as a rate method for assay of the free hormone fraction. Only two set-ups were found to be suitable for this purpose, the symmetric and the infinite or pseudo-infinite volume type of arrangement. The other two types of processes are suited for free hormone fraction estimation only at equilibrium. However, also in these cases time course analysis may be very useful since it permits prediction of the time needed to attain equilibrium. The extent to which equilibrium is approximated may have a large bearing on the accuracy of the measurement result (appendix IIA).
The dialysis method of e.g. Meyer et al.(1961) in which serum, to which labelled cortisol was added, is dialysed against a solution containing human serum albumine of the same concentration as occurring in serum, may be considered as an example of the general dialysis procedure of which the time course is given by eq.(II-10). The method yields at equilibrium relevant information on the partitioning of cortisol over its binding proteins. Also here, it is interesting to know when equilibrium will be attained for given values of f_1 and f_2.
With help of eq.(II-10) it can be readily shown that dialysis against a binding protein solution will require a longer time to reach equilibrium than dialysis against buffer.

Numerous applications of both equilibrium dialysis procedures in which serum is dialysed against buffer, and tracer is added either to serum or buffer, have appeared in the literature. These methods

have been used both for ligand-protein binding studies and free hormone assay. Also here, dialysis rate measurement is not feasible.

The infinite volume type of dialysis in which a ligand and binding protein solution is dialysed against a continuously flowing stream of buffer is applied as "Dynamic dialysis" by a number of investigators for characterising the interacting of small molecules with macromolecules. As the ligand is gradually removed from its binding agents, one single sample permits assay of binding avidity at different ligand levels. Ligand release curves can be evaluated by various relatively complicated mathematical manipulations (Pedersen et al. (1977).
Infinite volume dialysis also has been applied for the measurement of free testosterone by a "Flow dialysis technique" (Moll et al. 1977). In this method, one compartment contains serum and added testosterone tracer and through the other (dialysate) compartment a constant flow of buffer is maintained. Equal portions of dialysate buffer are collected at the outlet of this half-cell. Very soon the concentration of labelled hormone in the dialysate fraction reaches a plateau, and the amount of tracer diffusing out per unit time is assessed. This is closely similar to Christensen's assay conditions in which also in the very first phase of the process the amount of tracer transferred is directly proportional with time and free hormone fraction. In fact eqs.(II-33 and 35) can be applied with slight modifications for Moll's method also. Restriction of the measurements to the very first phase is imperative not only because of the proportionality between tracer transferred and time like in Christensen's approach, but also to meet the requirements imposed by what in terms of the present study would be assumption 4. The latter obviously is not relevant to Christensen's method. In contrast to Christensen, Moll et al. measured the cell permeability constant by assessing the diffusion rate of tracer testosterone added without serum.

The general model for the time-course of dialysis processes and its dependence on the free hormone fraction was derived with the assumption that diffusion of tracer through the membrane is the only rate-controlling step in the sequence of processes that constitue

the transfer of a tracer molecule from one compartment to the other. Experimental experience indicated that under certain circumstances the transport of tracer in the solution outside the membrane might be rate-controlling too. Therefore the mathematical model for symmetric dialysis was extended to include this effect, which can be mathematically described by means of the film diffusion concept. The film diffusion, or diffusion layer, concept is no uncommon notion in electrochemistry and in the theory of ion exchange. It was from the latter field - more specifically the theory of ion-exchange membranes - that the principle was adopted. The steady state flux equation for a non-electrolyte through an ion-exchange membrane (Helfferich 1962) was modified to include complex-formation of the diffusible species with a substance that has no access into the membrane. Furthermore the infinite volumes were replaces by finite volumes.

In addition to the explanation of departures from proportionality between dialysis rate and free hormone fraction, the film diffusion concept might have other important implications. This can be made plausible when the flux equation (II-21) is modified in such a way that in compartment 2 the free hormone fraction is infinitesimally low as compared to $f_1 = f$ in the other compartment. In fact this is the pseudo-infinite volume model. Because of this very low f_2, only the diffusion layer in compartment 1 influences the flux, so that one obtains, after elimination of c':

$$J = \frac{fc}{\frac{h}{D_m} + \frac{f\delta}{D_\ell}} \qquad \text{(II-36)}$$

The above model may be considered as a rough approximative description of a target cell for a particular hormone, immersed - either in vitro or in vivo - in a solution in which hormone concentration is kept constant.

A very rapid transport or metabolism of the hormone at the moment it appears on the inside of the plasma membrane is represented here by $f_2 = 0$. This model has some very interesting properties. As already known, for conditions of pure membrane diffusion control

(D_m, δ, f << and/or h, D_l >>) so that the second term in the denominator disappears, the flux of hormone into the cell is proportional to fc, i.e. the free hormone concentration in the medium. When opposite conditions prevail, the first term in the denominator of (II-36) is deleted, and f will be dropped from the expression so that the flux becomes dependent exclusively on c, the total hormone concentration in the medium, regardless of free hormone concentration or fraction. In other words, the more the flow into the cell is controlled by diffusion in the solution adjacent to the membrane, the less the free hormone and the more the total hormone concentration is representative of the flow of hormone into the cell.

It is tempting to speculate on a posssible physiological significance of the phenomena predicted by this model. The following general observations would be consistent with a role for the film diffusion effect:

1. The biological effect of strongly bound hormones is better correlated with free than with total hormone concentration, and the reverse tends to be the case with weakly bound hormones (e.g. thyroxine vs. aldosterone).

2. Since low flow rates promote film diffusion control and strong binding counteracts it, hormones that are weakly bound in serum display their action by preference in tissues in which blood flow is high, whereas strongly bound hormones tend to act everywhere, also in less strongly perfused parts of the body.

3. Changes in binding protein levels while homeostasis is maintained may in some instances be accompanied by changes of free hormone concentration. E.g. when f is increased as a consequence of lowered binding protein concentration, eq.(II-36) predicts, at a constant flux, an increase of the numerator, if the film diffusion term in the expression is significant. The conventional hypothesis does not take into account the possible effect of film diffusion and therefore predicts constancy of the free hormone concentration regardless of binding protein level.

Other important consequences may be expected if film diffusion actually would play a role at the surface of the living cell. When in vitro the uptake of hormone, from a medium that does not contain binding protein, into cultured cells is measured, the process may be largely diffusion controlled (Krenning et al., 1979). If binding protein is included in the medium so that f will decrease from unity to a certain value, the film diffusion term will be smaller and very probably the process will be less controlled by diffusion. When the uptakes under these two different conditions are compared with the free hormone concentrations in the medium, one would find that in the presence of binding protein the uptake is higher than expected in the basis of the uptake from the protein free medium and the free hormone concentration measured in the protein containing medium, so that it may seem that a larger proportion of total hormone is involved in transport into the cell than the proportion that is unbound. Also this can be read from eq. (II-36):
at equal free hormone concentration (fc) the flux is higher at lower values of f.
As a conclusion it seems justified to take the film diffusion concept into consideration as a possibility for explaining some poorly understood phenomena concerning cellular uptake of hormones.

It has been suggested that the rate at which steroid hormones dissociate from their binding proteins may be characteristic of their biological role (Westphal 1977). Thus, the serum binding proteins must be able to provide a rapid supply of hormone to its target cells, whereas intracellulair receptor proteins, which themselves depend in their action on association with steroid hormone, release the hormone only slowly. However, the (equilibrium) dissociation constants of specific serum binding proteins and specific intracellular receptors are of the same order of magnitude. A further role for the dissociation rate constants was suggested by Robbins (1974) in determining the relative importance of the thyroxine binding proteins in serum for the supply of hormone to the cells, by taking into account the proportions of hormone bound to each protein species and the differences in dissociation rate constants.

A possible dependence of cellular uptake of hormone on the dissociation rate of hormone serum protein complexes lately has been considered as improbable in view of the amounts of free hormone that can be generated per unit time and the actual disposal rates (e.g. Hillier 1975). However, very recently it was suggested (Ekins 1979) that there may be sites, in blood capillaries for example, where the total (and hence also the bound) concentration of hormone is lower than in the main stream because supply of hormone by blood flow in these small vessels lags behind the hormone uptake in the tissue. From the lower concentration of hormone-protein complex, less free hormone can be generated and it might be even so that dissociation would become rate-limiting. In that case, bound rather than free hormone would determine the flow of hormone into the cells. Local lowering of total hormone concentration is readily conceivable in view of the film diffusion concept, and therefore it seems reasonable to infer that in principle, film diffusion rate control may lead additionally to dissociation rate control. Whether this indeed has any biological significance can be judged with more certainty only after development of a model for hormone transport through a membrane which includes the mutual influence of film diffusion and dissociation rate control and when more and reliable data on in vivo hormone flux into cells are available.

II.6. Summary

Chapter II deals with the presentation of the symmetric dialysis method for free hormone assay within the setting of other, mostly well-known, dialysis methods for free hormone assay or binding studies.
Because of the intrinsic limitations inherent in equilibrium dialysis as discussed in ch.I, the various dialysis methods are now considered as rate processes in order to investigate whether the rate, at which the equilibrium distribution of tracer is approached, may serve as a means for assessing the free hormone fraction, rather than the equilibrium distribution itself. A general mathematical model is derived which comprises all procedures

considered as particular cases. This model gives a relation between free hormone fractions in each compartment and the distribution of tracer over the two compartments as a function of time (II.2). The (fractional) dialysis rate is defined as the natural logarithm of the ratio of free tracer concentration differences at $t = 0$ and $t = t$, divided by time. For symmetric dialysis the ratio of free concentration differences is equal to the ratio of total tracer concentration differences so that the dialysis rate is readily assessible. The dialysis rate is a time-independent parameter.
Some assumptions were necessary for the derivation of this model. Since in symmetric dialysis identical samples are present on both sides of the membrane and the process consists only of redistribution of tracer hormone, the assumptions concerning constancy of all concentrations involved are readily fulfilled. Together with the assessibility and the direct proportionality with the free hormone fraction of the dialysis rate, this makes symmetric dialysis the rate method of choice (II.3). In addition, precision can be made high and independent of the measured free hormone fraction, such in contrast to equilibrium dialysis. Worth mentioning is the much slower rate of equilibration.
For actual measurement of free hormone fractions some constants (membrane permeability and area, compartment volume) of the dialysis equipment or at least their product must be assessed. A reference solution with known free hormone fraction serves that purpose.

The mathematical model which forms the theoretical fundament for the symmetric dialysis method is based on the assumption that of the processes involved in transfer of tracer from one compartment to the other, only the diffusion of free tracer through the membrane is rate-determining. Other processes involved (transport of tracer through the solution towards the membrane and dissociation of tracer from its binding proteins) are assumed to be infinitely fast.
This assumption is further examined in II.4, in which the mathematical model is derived first in a more general way without assumptions concerning the rate-controlling step (II.4.1). Transport of tracer in solution is described in terms of the film diffusion concept (II.4.2) which makes it possible to bring the

convection and diffusion processes involved in a mathematical form and to explain the non-linearity of dialysis rate and free hormone fraction sometimes observed in practice. In II.4.3 an expression for the dialysis rate is derived in which also the forward and backward rate constants of the complex formation of hormone and binding protein occur. By means of this expression it is possible to predict under what circumstances - which are not too likely - dissociation or association rates may influence the overall dialysis rate.

Finally rate methods from the literature are briefly discussed and a possible biological significance of the described rate-limiting factors is considered (II.5).

Appendix II. The time course of some non-symmetric dialysis processes.

By substitution of the appropriate values for f_1, f_2, V_1, V_2 in the general formula (II-10) the time course of other types of dialysis processes can be derived.
For type 1) dialysis ("conventional" equilibrium dialysis) $f_2 = 1$ since no binding protein is present in compartment 2. For simplicity of notation $f_1 = f$ and further it is assumed that $V_1 = V_2 = V$. Consequently, eq.(II-10) reduces to:

$$\ln Q_t = \ln \frac{fc_1 - c_2}{fc_0} = - \frac{f + 1}{V} A D t \qquad \text{(II-A1)}$$

Observation of the right hand member shows that for very low values, f virtually does not influence the dialysis rate.
Since $0 < f \leq 1$ the dialysis rate cannot change by more than a factor 2.
Inspection of the left hand member shows that the dialysis rate cannot be assessed experimentally because it contains f which is just the quantity to be measured.
This is equivalent to stating that it is not possible to determine how far the process has advanced towards equilibrium because the tracer distribution at equilibrium is not known.

In case 2) tracer still migrates from compartment 1 to compartment 2 but now the latter contains the binding protein solution, so that now $f_1 = 1$ and $f_2 = f$. Still, $V_1 = V_2 = V$. Substitution in eq.(II-10) gives:

$$\ln Q_t = \ln \frac{c_1 - fc_2}{c_0} = \frac{f + 1}{V} A D t \qquad \text{(II-A2)}$$

This shows that with regard to response and assessibility the same applies as for case 1). Also the half-lifes and the final tracer distribution are equal. The important and interesting difference between these processes originates from the fact that final equi-

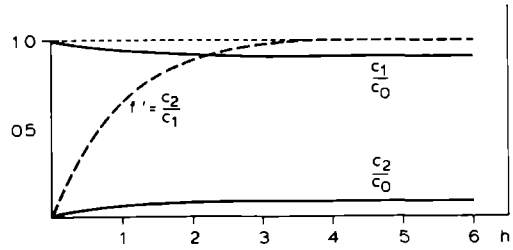


Fig.IIA.1.

Graphical representation of eq.(IIA-1) for the case that f = 0.1. Tracer concentrations are shown relative to total tracer present. After 4 hrs f" has become virtually equal to its equilibrium value. (Scale to be divided by 10.)

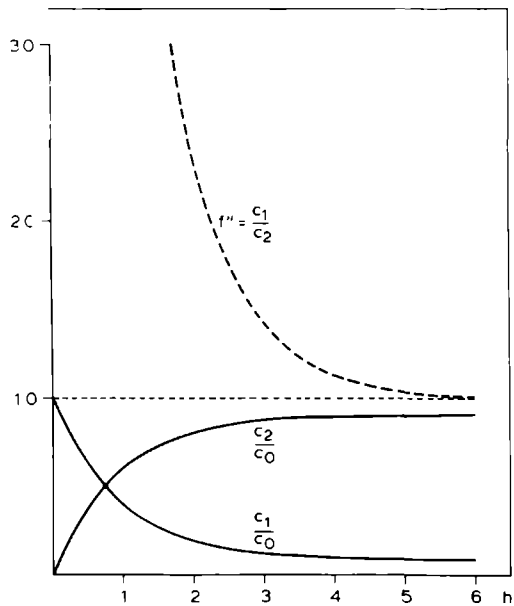


Fig.IIA.2.

Graphical representation of eq.(IIA-2) for f = 0.1. After 4 hrs f" is less close to its final value than in the previous case, so longer dialysis periods are needed for accurate measurement.

librium is approached from different directions (figs.II.1, IIA.1, 2). The equilibrium concentrations in the protein-free solution (dialysate, compartment 2 in case 1) and compartment 1 in case 2)) are identical for the two processes and equal to $c_0 f/(1 + f)$. The initial concentrations are 0 and c_0 respectively so that the length of the pathway towards equilibrium is different. So if both processes have covered e.g. 99% of this route, the dialysate concentrations generally will not be equally close to the final equilibrium concentration.
At 99% of equilibrium $Q_t = 0.01$ so that (eqs.IIA-1, 2):

$$fc_1 - c_2 = 0.01\ fc_0$$

and

$$c_1 - fc_2 = 0.01\ c_0$$

for type 1) and 2) equilibrium dialysis.
If $f'' = c_2/c_1$ and $f'' = c_1/c_2$ are used as estimates of f in type 1) and 2) processes respectively, which is allowed according to the assumptions in II.2., one obtains:

$$f'' = \frac{c_2}{c_1} = \frac{0.99\ f}{0.01\ f + 1}$$

and:

$$f'' = \frac{c_1}{c_2} = \frac{0.01 + f}{0.99} = 1/99 + 1.01\ f$$

In the first case, f" assumes a value between 0.98 and 0.99 f, but in the latter case there is a constant contribution of 0.01 (1/99) to the value of f". The consequences hereof are best explained with help of the following example:
Let f be 0.01. Then at 99% of equilibrium one finds by calculation from the above formulae for the first case that f" = 0.009899.

At the same time one finds in the other case that f" = 0.0202, being about twice the value of f.
In order to obtain also here an error of about 1%, the same as found for the first case, f" = c_1/c_2 must be about 1.01% from which can be calculated by means of eq.(IIA-2) that Q_t has to be 0.0001 instead of 0.01. Substitution of these values of 0.01 and 0.0001 for Q_t in eqs.(IIA-1) and (IIA-2) respectively and assuming for AD/V a value of 1 (expressed in hours) thereby using the same order of magnitude as observed with the actual experiments (cf.V), one finds that in order to attain an accuracy of 1% one needs a dialysis time of 4.6 hrs. for the "conventional" and about twice as long (9.12 hrs) for the "reverse" equilibrium dialysis processes. It must be noted that this difference in time required will be much less marked at higher values of f. When f = 0.5 it is not important any more from which side equilibrium is approached since the distance to be covered is equal. When f >0.5 the reverse situation arises, in which the final concentration in the dialysate is reached later when tracer is added on the protein side.

Type 3) dialysis (infinite volume) is characterised by $V_1 = V$, $V_2 = \infty$, so that $c_2 = 0$. Furthermore, $f_1 = f$. The pseudo-infinite volume process leads, with $f_1 = f$ and $f_2 = 0$ but $V_1 = V_2 = V$, to the same rate equation when substituting these conditions in eq. (II-10):

$$\ln Q_t = \ln \frac{c_1}{c_0} = - \frac{f}{V} A D t \qquad \text{(IIA-3)}$$

This shows that the dialysis rate $U = -1/t \ln Q_t$ is directly proportional to f. It is also directly assessible because f has been dropped from the expression for Q_t. The time required for 99% of equilibrium, again taking f = 0.01 and AD/V = 1 is 460 hrs. and the half-life is 69.3 hrs.

CHAPTER III

METHODS

III.1. Handling of dialysis equipment and measurement data (general).

III.1.1. Dialysis equipment.

All dialysis experiments were carried out in a "Dianorm (Diachema A.G. Rüschlikon, Switzerland) equilibrium dialyser". This device consists of a rotating apparatus in which twenty dialysis cells can be mounted in order to incubate with constant agitation. The dialysis cells are of Teflon and consist of two parts between which the membrane is fitted. Access to the compartments is provided by three holes for each half-cell. This device (fig.III.1) ensures constant membrane area and compartment (not solution) volume besides fast and reproducible filling and emptying of the cells. Constant temperature can be provided either by a thermostatted cupboard or a water bath. Rotation speed is adjustable (4, 8, 12, 16 or 20 rpm.). Mainly two types of cells were used. The one type has a working volume of 1.0 ml and a membrane area of 4.5 cm^2. The other type of cell has a working volume of 0.2 ml and a membrane area of 2.0 cm^2. Full specifications of cells are given in table III.1.:

Cell Type	Max. Volume (ml)	Working Volume (ml)	Min.Working Volume (ml)	Membrane Area (cm^2)
Micro 0.2	0.25	0.2	0.125	2.0
Macro 1	1.36	1.0	0.7	4.5

Table III.1

Membranes either were cut from Spectrapor 2 or Visking dialysis tubing or ready made Diachema membranes were used.
Molecular weight cut-off values for Spectrapor 2 and Diachema were 12,000 - 14,000 and 5,000 respectively. For the Visking dialysis tubing it is not specified but it is assumed to be somewhere about 20.000.

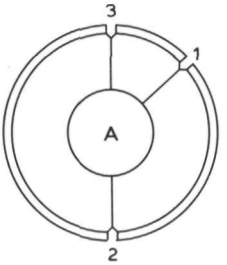


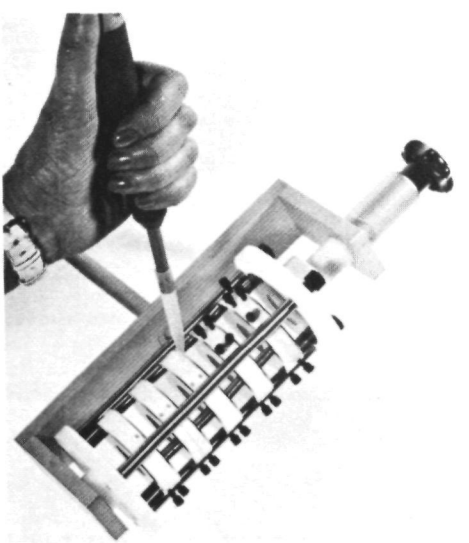
Fig.III.1.

Side-view of a dialysis half-cell.

A. Membrane area
1. Filling hole
2. Outlet hole
3. Deairation hole

Fig.III.2.

Filling operation.



Prior to use the membranes are immersed in distilled water which is frequently changed and subsequently assay buffer is used for rinsing. They are stored at 4^{o} C in buffer for up to 14 days. Just before the membrane is placed in the cell it is briefly blotted between tissue paper, so that its humidity is just adequate to permit its fitting smoothly and firmly in its place. The cells are assembled five by five in cell carriers in which pressure is applied to all five cells at a time by spring loaded spacers to prevent leaking (fig.III.2).

The cells are filled through the filling hole, by means of an Eppendorf disposable pipet tip (fig.III.2), while keeping the deairation hole open and the outlet closed. After closing the holes with plastic stoppers, the cell carriers are placed in the rotating apparatus. For equilibrium dialysis, the process is started simply by turning on the rotating device. Since the result depends on the temperature at equilibrium only, it is not essential to bring cells and solution to the assay temperature beforehand. Tracer solution, if employed, may be added together with the assay solutions or separately into the cells. For symmetric dialysis, the temperature should be constant during the complete process. This is accomplished for most of the experiments to be described by leaving the rotating device with filled cells run for temperature equilibration for at least half an hour before addition of tracer. In most instances tracer is added by means of a repeating dispenser for 10 µl (Hamilton) or less often a precision 10 microliter syringe (S.G.E.) is used. In both cases the needles are provided with spacers made from flexible polyethylene tube in order to prevent perforation of the membrane. Alternatively, tracer may be added to the assay solution before filling the cells. In that case, both solution and cells must be at the assay temperature before filling. Obviously with dialysis times exceeding 24 hours this temperature equilibration is less important. After dialysis small pieces of polyethylene tubing are put in the outlet holes. Receptacle tubes are placed over the outlets thus obtained and the complete cell carrier and tubes are inverted. Emptying of the cell halves is done by removal of the stoppers from one of the upper holes and careful blowing

through of some air e.g. by means of an empty Eppendorf pipet of a volume exceeding that of the cell. By this procedure a minimal time lag between emptying the first and the last cell is provided. Aliquots from the cell contents are taken for radioactivity measurement or for further treatment.
The cells are cleaned first by rinsing the assembled cells with a few ml of water after which the cells are taken apart, the membranes discarded and the cell parts placed in small racks and cleaned by running tap water for a few hours. Afterwards the cell halves are thoroughly rinsed with distilled water, especially the holes, and dried. Occasionally more thorough washing is applied using a special detergent (Decon 90) and ethanol for the final rinsing step.

III.1.2. Equilibrium dialysis.

Basically there are two ways of estimating the concentration of free hormone by means of equilibrium dialysis: the direct method, in which free hormone is (radioimmuno-) assayed directly in the dialysate after equilibrium has been reached, and the indirect method, in which radioactive labelled hormone is added before dialysis. From the tracer distribution at equilibrium over the two compartments an estimate of the fraction of hormone that is free is computed which subsequently is multiplied by the total hormone concentration in order to obtain the free hormone concentration.

III.1.2.1. The direct method.

The relevant measurable quantities for the direct method are:

F = concentration of free hormone in the dialysate (at equilibrium)
H_0 = total hormone concentration in the original sample

Known are:

V_0 = the volume of original sample actually present in the dialysis cell

V_t = total volume of dialysis system (left + right compartment)

The purpose of the assay is measurement of:

I_0 = true concentration of free hormone in the original sample

It is not possible to measure the true free hormone concentration corresponding to the original sample directly by equilibrium dialysis (I.5). Instead it may be approximated by means of the quantity F_0' which is calculated according to eq.(I-14):

$$F_0' = H_0/(H_0/F - V_t/V_0 + 1) \qquad \text{(I-14)}$$

F_0' may very closely approach the true value of F_0 and even may be equal to F_0 under certain conditions that are discussed in ch.IV.2.1.
A practical example is the following (data from IV.4.2.):
Total cortisol is assayed in a certain serum, which gives H_0 = 582.5 nmol/l. Twice diluted serum (0.150 ml) is dialysed against an equal volume of buffer, which means that V_t = 0.3 ml and V_0 = 0.075 ml. After equilibrium has been reached, cortisol is assayed in the dialysate: F = 40.6 nmol/l.
Application of eq.(I-14) gives F_0' = 51.3 nmol/l.
When undiluted serum is dialysed instead, V_0 = 0.150 ml and V_t is still 0.3 ml. Now F is found to be 54.6 nmol/l and application of (I-14) gives F_0' = 60.2 nmol/l. The latter is a closer approximation of the value obtained by symmetric dialysis of undiluted serum (68.4 nmol/l, see V.3.3.,.
This demonstrates that the approximation is better, the lower the value of V_t/V_0.

III.1.2.2. The indirect method.

The relevant measurable quantities for free hormone assay by an indirect equilibrium dialysis assay are:
H_0 = total hormone concentration in the original sample

c_1 = concentration of radioactivity (e.g. cpm/ml) in the dialysand at equilibrium

c_2 = concentration of radioactivity in the dialysate

V_0 = volume of original sample present in the dialysis system

V_0' = original dialysand volume

V_t = total volume of the dialysis system

c_0 = concentration of radioactivity in the original sample e.g. if $\underline{c}$ cpm are added, $c_0 = \underline{c}/V_0$

V_1 = volume of dialysand at equilibrium

V_2 = volume of dialysate at equilibrium

The radioactivities are assumed to be corrected already for efficiency differences and to represent entirely the intact tracer hormone, without contamination. V_1 and V_2 can be assessed by total protein assay in the sample before and the dialysand after dialysis, which gives the ratio V_1/V_0. Since V_0 is known and $V_1 + V_2 = V_t$ also V_1 and V_2 are readily computed. First the tracer recovery is assessed by means of eq.(IV-13):

$$\text{rec.} = \frac{c_1V_1 + c_2(V_t - V_1)}{c_0V_0} \cdot 100\% \qquad \text{(IV-13)}$$

If the tracer is quantitatively recovered, consecutive measurements in the same free hormone assay can be confined to assessment of c_2 and c_0 only (V_0 and V_t known). In that case, the free hormone fraction f (free hormone concentration divided by total hormone) corresponding to a dilution of the sample for V_0 to V_t is calculated by means of (IV-3):

$$F = c_2V_t/c_0V_0 \qquad \text{(IV-3)}$$

Multiplication of f by the total hormone concentration belonging to that dilution (i.e. $H_0.V_0/V_t$) gives the corresponding free hormone concentration F.

An estimate f_0' of the free hormone fraction f_0 in the original sample can be obtained by application of (IV-4):

$$f_0' = ((c_0/c_2) - (V_t/V_0) + 1)^{-1} \qquad \text{(IV-4)}$$

Multiplication of f_0' with H_0 gives an estimate F_0' of the free hormone concentration for the original, undiluted sample which is identical to F_0' dealt with in paragraph III.1.2.1. As in the direct method, F_0' is only an approximation of F_0 but it is equal to F_0 under certain conditions (ch.IV.2.2.), and it is a better approximation at lower values for V_t/V_0.

If the tracer is not quantitatively recovered, the radioactivity in the dialysand (c_1) also has to be used. The same quantities f and f_0' as given above can also be calculated using the following formulae:

$$f = c_2V_t/(c_1V_1 + c_2V_2) \qquad \text{(IV-5)}$$

$$f_0' = (V_1/V_0)(c_1/c_2 - 1) + 1)^{-1} \qquad \text{(IV-9)}$$

These formulae necessitate measurement of V_1/V_0 in addition to c_1 and c_2.

The following free cortisol assay may serve as a practical example: 1 ml of 1.333 times diluted serum is dialysed against 1 ml buffer, so that V_0 = 0.75 ml and V_2 = 2.0 ml. A small volume (10 µl) of ^3H cortisol tracer is added to the cell and also to 0.75 ml serum and to 0.75 ml buffer. Of the serum a 0.1 ml aliquot is counted (18056 cpm/ml) and of the buffer 0.1 ml and 0.5 ml aliquots are counted for radioactivity (18430 and 17051 cpm/ml)*. If the counting efficiency for 0.1 ml buffer is taken as unity the relative counting efficiencies are 0.9797 (0.1 ml serum) and 0.9252 (0.5 ml buffer). After dialysis equilibrium is reached, 0.1 ml aliquots of dialysand and 0.5 ml aliquots of dialysate are taken and radioactivity is

* These additional tracer countings and the further correction procedure for differences in counting efficiency evidently is unnecessary when counting equipment with automatic efficiency correction is used.

measured (12293 and 1203 cpm/ml respectively). Also the dilution of the sample in the dialysand, V_1/V_0 is assessed (1.389) by means of total protein assays in the original sample and in the dialysand at equilibrium. The concentration of radioactivity in the dialysand is now calculated as follows: $12293\ (1 - (1 - 0.9797)/1.389)^{-1}$ = 12475 cpm/ml, thus taking into account the counting efficiency in the dialysand which differs from that in pure serum because of the dilution that has occurred first by placing the sample into the cell in 1.333 times diluted form and later during dialysis by fluid shift due to osmotic pressure. Dialysate radioactivity is calculated as 1203/0.9252 cpm/ml. In a separate experiment was found that 1.5% of the tracer radioactivity did not bind to cortisol antibody or serum proteins. So of the added tracer 0.75 x 18430 x 0.015 = 207 cpm is unbound, non-cortisol radioactivity which is freely distributed over both dialysis compartments (2 ml in total) so that it acquires a concentration of 104 cpm/ml. This number must be subtracted from the efficiency-corrected dialysand and dialysate radioactivities in order to obtain c_1 and c_2. This gives c_1 = 12371 and c_2 = 1196. In order to obtain c_0 one must subtract 207/0.75 cpm/ml from 18430 cpm/ml by which the set of data is completed.

c_0	= 18154	cpm/ml
c_1	= 12371	"
c_2	= 1196	"
V_0	= 0.75	ml
V_t	= 2	"
V_1/V_0	= 1.389	"
V_1	= 1.0417	"
V_2	= 0.958	"

The tracer recovery is obtained by employing eq.(IV-13) which gives a value of 103.1%. Assuming that this value represents quantitative recovery, both sets of equations (IV-3, IV-4) or (IV-5, IV-9) may be applied. One obtains the following results:

(IV-3) : f = 0.176

(IV-5) : f = 0.170

(IV-4) : f_0' = 0.0740

(IV-9) : f_0' = 0.0715

The implications of the various formulae used can be found in ch.IV.2.2., while more experimental material is presented in ch.IV.3. and IV.4.
It is useful to mention that c_2 and c_0 may be replaced by F and H_0 in formulae (IV-3) and (IV-4), since all concentration ratios are equal for labelled and unlabelled hormone as a consequence of the basic assumption that labelled and unlabelled hormone are equally distributed over free and bound forms. Thus it is also possible to obtain free hormone fractions f and f_0' by means of the direct assay method.

III.1.3. Symmetric dialysis.

In symmetric dialysis equal solutions are present on both sides of the membrane. The process is started with addition of tracer in the first compartment and it is ended by emptying the cell. From the concentrations of radioactivity in both compartments (c_1 and c_2) a function of tracer distribution, ln I, is calculated where I is given by (see ch.II.3.):

$$I = (c_1 - c_2)/(c_1 + c_2)$$

The dialysis rate was defined by $U = -(1/t)\ln I$, so that it can be obtained simply by division of -ln I by the dialysis time. A more accurate estimate can be obtained by assessment of ln I at different pointsin time. Then, the slope of a -ln I vs. t curve which can be assessed by linear regression analysis, gives the dialysis rate U for any free hormone assay, linearity of this curve is a necessary requirement for the applicability of symmetric dialysis. More information about such "time curves" can be found in ch.V.2. When setting up an assay, first the relation between dialysis rate U and free hormone fraction f, which ideally is a direct proportionality, must be assessed. The dialysis rate for a number of reference standard solutions with known f lying in the range of interest should be determined and plotted against f. Also the dialysis rate in pure buffer (i.e. f = 1), must be determined. There

are three possibilities for the U vs. f curves, which are schematically depicted in fig.III.3. In the first two cases, the range of interest is located on a linear section of the U vs. f standard curve. In these two cases the use of a single standard solution with a value of f in the linear range is sufficient for assessing unknowns. Let the unknown free fraction be f_x, the dialysis rate U_x and the free fraction and dialysis rate of the reference solution f_r and U_r. Then, by virtue of the direct proportionality of U and f in the measurement range:

$$f_x = (U_x/U_r) f_r$$

Often the standards and unknowns will be dialysed during exactly the same period of time, so that U_x and U_r may be replaced by ln I_x and ln I_r. In the ideal case of fig.III.3a direct proportionality between U and f exists up to f = 1, so that pure buffer may be used as a reference standard (f_r = 1). The ratio U/f is called the cell permeability factor since it is a function of membrane and cell properties (see eq.II-23). In the case of fig.III.3a up to f = 1 it is a constant (eq.(II-14) then holds) which is equal to U for pure buffer. When the range of f values in which unknowns have to be determined falls in the non-linear part of the curve (fig.III.3c), the only possible approach to the estimation of F is

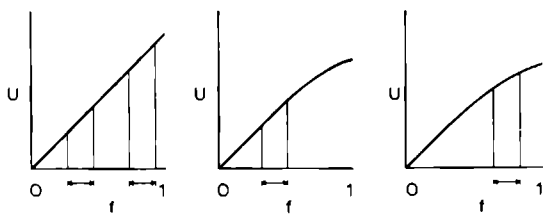


Fig.III.3. a b c

by construction of an U vs. f standard curve for that particular range using an adequate number of different standard solutions to cover that range.
A standard solution for use in symmetric dialysis must meet two requirements; it must be stabile with storage, and its true free hormone fraction must be assessible. In spite of the drawbacks of equilibrium dialysis, it may be applied with certain precautions for establishing true free hormone fractions of reference substances (ch.V.3.3).
Under favourable conditions (ch.V.3) one may obtain for free cortisol assay a linear curve as in fig.III.3a. With free T4 and free T3 the situation of fig.III.3b is obtained, while free aldosterone represents the more problematic case of fig.III.3c. It must be noted (ch.V.3.3) that linearity of the curve is enhanced by high rotation speeds and low filling levels of the cells. Further on abridged calibration procedures of single standards for T4, T3 and cortisol are given. Assay of free aldosterone by symmetric dialysis requires the use of more than one standard.

III.1.3.1. Assay of free thyroid hormones in 1 : 50 diluted serum.

By symmetric dialysis the free hormone fraction f_x of an unknown sample is obtained, which must be multiplied by the total hormone in order to obtain the free hormone concentration. Along with the unknowns, a serum standard with known free fraction f_r is assayed. This standard itself has been calibrated against a reference solution consisting of human serum albumin with added T4 and T3.

Procedure:

0.1 ml of sample or standard serum is diluted with 4.9 ml phosphate buffer. In two "macro" (1 ml) cells, 1 ml portions are pipetted. The filled cells are left to temperature equilibrate for at least half an hour with rotation. The process is started by pipetting into one half-cell 10 µl of ^{125}I-T4 or ^{125}I-T3 tracer solution, containing about 10.000 - 20.000 cpm, by means of a Hamilton re-

peating dispenser. The specific activity must not be below about 50 mCi/mg. Actually, T4 tracer with about 150 mCi/mg and T3 tracer of 900 mCi/mg has been used. For free T4 the process is terminated after overnight dialysis and for free T3 after two hours. In both cases the exact dialysis time is recorded. The cells are emptied as described in III.1.1 and 0.5 ml aliquots from each cell-half are counted for radioactivity. With these counts (c_1 from the compartment where tracer was added at the start and c_2 from the other compartment) a function of the tracer distribution (ln I) is calculated where $I = (c_1 - c_2)/(c_1 + c_2)$. Let I for an unknown be I_x and for the reference standard serum I_r, then the free hormone fraction of the unknown, f_x, is found by:

$$f_x = f_r \ln I_x / \ln I_r$$

For ln I_r the duplicate mean is taken to calculate single values of f_x.

Assay of reference standards (determination of f_r):

A primary standard for both free T4 and free T3 assay is calibrated first by the following procedure:
1 ml of 1% HSA solution in phosphate buffer containing 1.25 µg/ml T4 and 30 ng/ml T3 is dialysed against 1 ml of buffer. After equilibrium has been reached (at 7 hrs about 99.9% of equilibrium is attained for 1 ml cells with low f solutions (see appendix IIA) T4 and T3 are radioimmunoassayed in the dialysate (ch.III.2.1). If F denotes the concentration of (free) hormone in the dialysate and H_0 the original total hormone concentration in the HSA solution, the true free fraction corresponding to 0.5% HSA, which will be used as the primary reference solution, can be estimated by:

$$f_r = FV_t/H_0V_0$$

Where in this case V_0 and V_t are 1 and 2 ml. The albumin solution itself could be used to serve as a standard in each assay series but it is more convenient to have a secondary (serum) standard as well for this purpose. The free T4 and T3 fractions of this

secondary standard are determined in exactly the same way as the unknowns. Of course a larger number of replicates will be used. With the above approach one would obtain for unknowns the actual free fraction at 1 : 50 dilution. It is possible to correct the free fraction of the secondary standard for dilution so that the results of the unknowns also include the same correction. If the free fraction of the secondary standard at a dilution factor L (= 50 in the present case) is denoted by $f_{r(L)}$, then the free fraction for the undiluted state $f_{r(0)}$ can be estimated by:

$$f_{r(0)} = ((1/f_{r(L)} - 1)L + 1)^{-1}$$

This estimate is based on the assumption of a linear dilution effect (ch.I.4, ch.IV) and is equivalent to eq.(I-16).

Example:

The 0.5% HSA solution was found to have a free T3 fraction of 0.0814. Symmetric dialysis of this solution for 2 hrs together with poolserum in 1 : 50 dilution gave : c_1 = 6136, c_2 = 1887 cpm/0.5 ml for 0.5% HSA and c_1 = 6183, c_2 = 1948 for the poolserum, so that -ln I values were 0.6356 and 0.6522 respectively. If these (single) values would be taken to determine $f_{r(50)}$ one would obtain: 0.0814 x 0.6522/0.6356 = 0.08352. Correction of this value for dilution using the above formula, would give $f_{r(0)}$ = 0.00182.

III.1.3.2. Assay of free cortisol in undiluted serum.

Procedure

In the 0.2 ml ("micro") cells, 0.150 ml of serum or standard is pipetted on both sides of the membrane. On one side 10 µl of buffer is added and the process is started directly thereafter by addition of 10 µl of ^{3}H-cortisol tracer at the other side. The process is terminated between 1 - 1.5 hrs and the cells are emptied as already described. With this relatively short dialysis time it is recommended to record the exact times per cell holder of five cells, if larger series are to be assyed. Then, in order to elimi-

nate the effect of differences in dialysis time, the dialysis rates U_x and U_r, obtained by division of $-\ln I_x$ and $-\ln I_r$ by the time in hours, are used for further calculations, instead of ln I values. After emtying, 0.1 ml aliquots from all compartments are counted for ^{3}H-activity, and -ln I values and (if necessary), U values are calculated. Unknown free fractions f_x follow from:

$$f_x = f_r \ln I_x / \ln I_r = f_r U_x / U_r$$

Again ln I_r or U_r is the mean of replicate determinations.

Determination of f_r.

H.S.A. cannot be used to make a reference standard for free cortisol since it is a to weak binder for this hormone, so whole serum will be used instead. An amount of undiluted poolserum (0.15 ml) is dialysed against an equal volume of phosphate buffer. After equilibrium is reached (4 hrs are sufficient) the cells are emptied and cortisol is radioimmunoassayed in the dialysates as described in III.2.1. Also total cortisol is measured in the serum. If the dialysate cortisol concentration is denoted by F and total cortisol by H_0, then the same formula as given for the H.S.A. solution in III.3.1 may be used to estimate the true free cortisol fraction $f_{r(2)}$ for twice diluted serum. A secondary standard consisting of undiluted serum may be derived from it, by means of $f_{r(0)} = f_{r(2)} U_{r(0)} / U_{r(2)}$. Data taken from ch.IV, table IV.9 may serve as an example. In a poolserum total cortisol (H_0) was found to be 582.5 nmol/l. The concentration in the dialysate (F), obtained by dialysing 0.150 ml undiluted serum against 0.150 ml buffer, was 54.5 nmol/l (mean of duplicate dialysis and duplicate radioimmunoassay). Since V_0 = 0.150 ml and V_t = 0.300 ml, one obtains $f_{r(2)}$ = 0.187. Symmetric dialysis of this twice diluted serum simultaneously with the same serum in undiluted form (which may be considered both as an unknown or a secondary standard) gave after 1 hr dialysis $-\ln I_{r(2)}$ = 1.0167 and $-\ln I_x$ = 0.6194, which results in f_x = 0.114.

III.1.3.3. Assay of free aldosterone in undiluted serum.

Procedure

The assay itself is carried out in the same way as described for free cortisol, except that dialysis must last shorter, about 15 minutes. Therefore the accurate recording of dialysis times for the different cell holders and the use of the dialysis rate U instead of -ln I is mandatory in this case.

Reference standards.

For the free aldosterone assay a single standard is not sufficient because in serum containing only the normally occurring binding proteins, a direct proportionality between dialysis rate and free hormone fraction, which in the previous cases led to simple assessment of free fractions using only one standard, does not exist. A standard curve must be contructed, for which a number of sera with different free aldosterone fractions are required. This may be accomplished by choosing high and low cortisol sera. According to the notion that total cortisol is a major determinant of the binding of aldosterone to C.B.G. (Zager et al. 1976, de Man et al. 1979), the free aldosterone fraction would be inversely related to total cortisol levels. As the standard curve procedure has not been carried out in complete form until now, the latter must be yet considered as a suggested approach. The free aldosterone fractions in the standards are measured by equilibrium dialysis using the indirect method, e.g. 0.150 ml of serum against 0.150 ml of ^{3}H-aldosterone in buffer. By virtue of the linear dilution effect that holds in this case (chs.I.4 and IV) it is possible to estimate the true free hormone fraction in undiluted serum from the data obtained by this experiment. If c_0 is the concentration of radioactivity in the tracer solution and c_2 that in the dialysate, the free fraction of the reference standard f_r can be found from

$$f_r = c_2/(c_0 - c_2)$$

This formula is a particular case of eq.(IV-4) and it is valid only in the case of equal volumina of sample and buffer, in other words,

when $V_t/V_0 = 2$. For c_0 and c_2 the counts in e.g. 0.1 ml aliquots from tracer solution and dialysate may be used.
The dialysis rates U_r of the standards are measured and a U vs f standard curve is constructed, on which free fractions of unknowns can be read, by means of their dialysis rates U_x.
As already stated, this multiple standard approach for free aldosterone has not been applied in practice until now, so it must be considered as tentative. Besides, it seems that equilibrium dialysis for free aldosterone, when only naturally occurring binding proteins are present, is the more straightforward and accurate method.

III.2. Miscellaneous methods used in chs.IV and V.

III.2.1. Radioimmunoassays.

Aldosterone.

Total aldosterone was measured in 1 - 3 ml plasma by radioimmunoassay after extraction and paper chromatography according to de Man et al.(1979).

Cortisol.

Total plasma cortisol was measured in the equivalent of 1 µl of plasma after heat and ethanol treatment. For measurement in plasma 0.05 ml is added to 1 ml 5% ethanol. After heat denaturation 0.1 ml of this solution is assayed. For measurements in dialysates a solution consisting of 0.1 ml dialysate, 0.25 ml ethanol 15%,0.4 ml aqua dest. and 7.5 µl of charcoal-treated, steroid-free plasma was prepared from which a 0.1 ml aliquot was assayed. Thus, the amount of dialysate assayed corresponded to 13.2 µl of plasma, which was taken into account when calculating free cortisol values.

Thyroxine (T4).

Total T4 was directly assayed in 5 µl samples. The antiserum used has a dissociation constant for T4 of 1.1×10^{-11} mol/l. About 100 pg of T4 is added in the form of tracer. Total incubation volume is 0.305 ml in barbital buffer containing 0.07% ANS to in-

hibit T4-TBG binding. Separation of bound and free is accomplished by the addition of 1 mg γ-globulin in 0.05 ml buffer followed by 0.5 ml 30% PEG. After centrifugation the supernatant is aspirated and the pellet counted.

Triiodothyronine (T3).

Total T3 was measured directly in 0.1 ml serum samples. The antiserum which is added to obtain a final dilution of 1 : 12500 has a dissociation constant for T3 of 1.2×10^{-11} mol/l. It had been raised against a T3-BSA conjugate, obtained by carbodiimide condensation. About 20 pg per tube of T3 is added in the form of ^{125}I-tracer. The total incubation volume is 0.55 ml in barbital buffer containing 0.25% BSA and 0.1% Thiomersal to block T3 binding to TBG. Separation of bound and free is done by means of the Double Antibody Solid Phase (DASP) method. 0.1 ml of DASP suspension is added in 0.5 ml buffer and incubated after which the solid material is spun down, washed and counted for radioactivity.

The T4 and T3 assays were applied for measuring the free hormones in 0.1 ml aliquots from equilibrium dialysates of HSA solutions of various strengths, containing 1.25 µg T4 and 30 ng T3 per % HSA.

III.2.2. Separation techniques.

T3 and T4 purification on Sephadex G 25 was carried out according to Ködding (1975). Separation of intact T3 or T4 tracer after dialysis was done by means of "Immophase" T4 or T3 antibody coupled to glass beads which are available as a suspension. 1 ml portions of the suspension were added to aliquots of varying size from the cells. Three washes with 1 ml of buffer were performed to remove non-antibody-bound radioactivity.

III.2.3. Statistical methods.

The following statistical operations were carried out according

to W. Mendenhall, Introduction to Probability and Statistics 1979, Duxbury Press, North Scituate, Mass.:

a. Linear regression analysis, including prediction and confidence intervals for the found relations and confidence intervals for slopes and intercepts.
b. Pearson correlation coefficients and significance tests.
c. One-way analysis of variance (Anova).
d. Simultaneous pairwise comparisons with protection level (Newman-Keuls procedure).
e. Confidence intervals for variation coefficients.

Propagation of errors was studied according to H.D. Young, 1962, Statistical Treatment of Experimental Data, Mac-Graw-Hill, New-York. Two-way Anova: R.F. Hirsch, Analysis of Variance in Analytical Chemistry, Anal.Chem. 49, 691A (1977).

- A.N.S.: 8-anilino-1-naphtalene sulfonic acid (Sigma)
- Aldosterone, tritiated, 40 - 60 Ci/mmol (New England Nuclear). Solved and diluted in ethanol. Evaporation and dissolution in phosphate buffer immediately before use.
- Barbital buffer, pH 8.6, 0.075 M: 12.4 g sodium diethylbarbiturate (Merck), 2.7 g diethylbarbituric acid (Merck) in 1 l distilled water.
- B.S.A.: Bovine Serum Albumin (Sigma).
- Cortisol, tritiated, 40 - 60 Ci/mmol (New England Nuclear). Handling as for ^{3}H-aldosterone, see above.
- DASP: Double Antibody Solid Phase; cellulose-coupled anti-rabbit IgG antiserum (Organon).
- H.S.A.: Human Serum Albumin, "reinst" grade (Behring).
- Immophase T4, T3: Antibody against T4 or T3 coupled to glass beads, suspension (Corning).
- Membranes: Diachema, M.W. cut-off 5,000 (Diachema); Visking dialysis tubing 44 mm (Union Carbide); Spectrapor 2, M.W. cut-off 12,000 - 14,000 (Spectrapor, not available any more in the Netherlands).
- Norit A (Fisher), 14 g charcoal/100 ml poolserum with overnight stirring strips thyroid hormones and steroid hormones from the binding proteins.
- P.E.G.: polyethylene glycol 6000 (Merck).
- Phosphate buffer, pH 7.4, 0.15 M: 4.92 g potassium dihydrogenphosphate (Merck) and 9.85 g di-potassium hydrogenphosphate (Merck) dissolved in 1 l aqua dest.
- Propanediol: Propane 1,2 diol, propylene glycol (BDH).
- Sodium azide (Merck).
- Sodium hydroxide (Merck).
- Thiomersal (BDH).
- Thyroxine, free acid (Sigma), stock solution of 20 mg/100 ml made in 0.1 N NaOH. Further dilutions in buffer.

- l-3,5,3'-triiodothyronine, free acid (Sigma). Solutions as for thyroxine.
- Thyroxine (T4)-^{125}I tracer, 150 mCi/mg (New England Nuclear) batches of 150 µli/0.5 ml diluted 80x with propanediol/water 1 : 1.
- Triiodothyronine (T3)-^{125}I tracer, 1000 mCi/mg (New England Nuclear), see further ^{125}I-T4.

Chapter IV

FREE HORMONE ASSAY BY EQUILIBRIUM DIALYSIS: AN UPDATED APPROACH TO THE EVALUATION AND INTERPRETATION OF EQUILIBRIUM DIALYSIS DATA.

IV.1. Introduction.

When using equilibrium dialysis for separation of free and bound hormone, there are in principle two possible approaches to free hormone assay: the direct method in which the concentration of unbound hormone is (radioimmuno-) assayed directly in the dialysate, and the indirect method in which the free hormone fraction is determined first with help of added tracer hormone. Multiplication of the result by the total hormone concentration yields the required value.

This indirect approach is most often a practical necessity since for many hormones the available assays are still not sensitive enough to measure the sometimes very low free hormone levels. Whatever approach is used, the aim is to obtain an as good as possible estimate of the free hormone concentration in undiluted serum. Since application of equilibrium dialysis, as was shown before (ch. I.5), always influences the original equilibrium in the same way as mere dilution would, this has to be done from data obtained in a diluted state.

As a rule, the available data on dissociation constants and binding site concentrations are too scarce or inaccurate to permit accurate calculation of the original free hormone concentration from actual measurements. Hence one must rely on simplifying assumptions concerning the relation between measured and "true" values. For the direct method, this relation is a matter of dilution effect only. There will be no misunderstanding about what is the concentration of free hormone in a dialysate when it is actually measured by radioimmunoassay. It should also be obvious that this concentration corresponds to a dilution of V_t/V_0. According to ch.I.5 definition of the free hormone fraction in a dialysis system for use in the

indirect method should also be no problem.
With this knowledge, straightforward methods for obtaining as good as possible approximations for the free hormone concentration or fraction in the original sample are derived in this chapter. This is done in IV.2.1 for the direct method and in IV.2.2 for the indirect method. The indirect method, which is most widely applied, is provided by the present approach with a set of alternatives and supplements.
Aspects of this approach are experimentally assessed by means of equilibrium dialysis experiments with aldosterone (IV.3) and cortisol (IV.4). The absence of any effect of dialysand dilution on the free hormone or tracer concentration and its consequences are verified in IV.3.1 and IV.4.1, while the effects of total dilution with (aldosterone) and without (cortisol) simplifying conditions are studied in IV.3.2 and IV.4.2. It is demonstrated (IV.4.3) that it is intrinsically impossible in the case of cortisol to measure the free hormone fraction for undiluted serum by equilibrium dialysis, such in contrast with the widespread opinion that Slaunwhite's calculation procedure applied on the dialysis of undiluted serum against buffer yields this parameter if dialysand dilution can be kept under control.

IV.2. Handling of equilibrium dialysis data.

IV.2.1. The direct method.

Hormone assay in a serum dialysate gives a direct estimate of F, the free hormone concentration that corresponds to the state in which the sample is diluted to a volume equal to that of the complete dialysis system. Generally one will be more interested in F_0, the unbound hormone concentration in the original, undiluted sample. A general relation between F and F_0 can be derived by eliminating H_0 from eqs.(I-6a) and (I-12b) but the result (eq.IC-1) is defined in terms of the binding parameters (P_i and K_i's) of the system, so it is not suitable for calculation of F_0 from the actually measured F. Furthermore F_0 is not explicit in this expression

which makes its evaluation difficult. Also some knowledge of the dissociation constants involved is required in order to judge whether a simple relation between F and F_0, which relies on certain limiting conditions, will hold. So, without knowledge of binding parameters, a single measurement of F gives no information on F_0 or on what formula to use to calculate F_0.
Therefore the following approach is suggested:

Measurements of F (F_1 and F_2) are made for at least two different dilutions. The first dilution should be as low as is technically feasible, and the second one should neither be too close to nor too far from the first one.
In addition to F_1 and F_2, H_0 is determined so that it is possible to calculate the values of F_{01}' and F_{02}' as estimates of F_0 by means of eq.(I-14). The following three combinations of results are possible:

1. $F_1 \simeq F_2$, i.e. in spite of the difference in dilution, F_1 and F_2 differ less than the assay method can detect. In this case, very probably F_0 is equal to F_1 and F_2 since the limiting condition for constancy of F (ch.I.4) appears to hold.

2. $F_1 > F_2$, but $F_{01}' \simeq F_{02}'$. The origin of this result is found by writing the latter identity in terms of a general expression for F_0' which can be obtained by inserting eq.(I-12c) in (I-14):

$$F_{01}' = H_0/(\Sigma\frac{P_{i0}}{K_i + F_1} + 1) \simeq F_{02}' = H_0/(\Sigma\frac{P_{i0}}{K_i + F_2} + 1) \qquad \text{(IV-1)}$$

which implies that:

$$K_i + F_1 \simeq K_i + F_2 \qquad (i = 1....n)$$

and since $F_1 > F_2$:

$$F_1, F_2 << K_i$$

Since F_0 still may be higher than F_1, there is no absolute certainty that this inequality also holds for F_0, which would imply,

according to I.4, that: $F_{02}' = F_{01}' = F_0$. However, since it was proven that $F/F_0 > V_0/V_t$ (appendix IC) it is most unlikely that any noteworthy error would result from assuming that also $F_0 << K_1$, especially when V_{t1}/V_0 is less than e.g. 2. Therefore, the assumption that $F_0 = F_{01}' = F_{02}'$ seems to be fully justified.

3. The above two possible results of the experiment both are consistent with the direct proportionality of the free to bound (f/b) ratio and dilution eq.(I-17), because of constancy of all $K_1 + F$ terms. When neither of the two conditions for this constancy (F constant; $F_0 << K_1$) is fulfilled, the following outcome of the experiment will be encountered:

$F_1 > F_2$ and $F_{01}' > F_{02}'$. Estimates of F_0 from such data can only be of a very approximative nature. With help of relations derived earlier (appendix IC), upper and lower limits for F_0 can be derived, but these may be rather wide apart.

From $F_0/F < V_t/V_0$ and $F < F_0' < F_0$ follows that:

$$F\ V_t/V_0 > F_0 > F_0' \qquad \text{(IV-2)}$$

Possibly more narrow limits can be found, but this will be a subject for future research.

In the present study, free hormone assay by means of the direct method has been actually applied only in the case of free cortisol. Values of F and F_0' for different dilutions, as presented in table IV.9 further on in this chapter, show that this system forms an example of case 3, so that no accurate estimate of F_0 can be made. An example of case 1 can be found in table IA.6 where F and F_0' values for free T4 are given, calculated for different dilutions. Since for low to moderate dilutions F and F_0' values hardly differ, F_0 would be very closely approached. Finally, when values of F and V_t/V_0 from table IV.3 are taken, and corresponding values for F_0' are calculated it is readily found that measurement of free aldosterone in serum by means of the direct method would be an example of case 2.

IV.2.2. The indirect method.

Measurement of the free hormone fraction by means of equilibrium dialysis with help of added radioactive labelled hormone is based on the assumptions that radioactive and non-radioactive hormone are distributed in the same way over the various orders of binding sites and the unbound form, and that the labelled hormone is present in real trace amounts so that its presence does not affect the equilibrium position of the system. After dialysis equilibrium has been reached, the buffer compartment contains tracer in free form and the serum compartment contains the same free tracer concentration in addition to bound tracer.
The following variables of the system at equilibrium are measurable or known:

c_1 = tracer concentration in dialysand
c_2 = " " in dialysate
V_1 = dialysand volume
V_2 = dialysate "

Quantities which are independent of equilibrium are:
c_0 = initial tracer concentration in original sample
V_0 = " sample volume
V_0' = " dialysand volume prior to dialysis
V_d = " dialysate " " " "
V_t = total (dialysate + dialysand) volume of the system
($V_t = V_1 + V_2 = V_0' + V_d$)
H_0 = total hormone concentration in original sample

The tracer concentrations c_0, c_1 and c_2 are expressed as amount of radioactivity (c.p.m. or d.p.m.) of the radiochemically pure substance (per unit volume). So in practice these quantities are obtained after purification or correction for radioactive contamination.
For a clear picture of the meaning of the above symbols the dialysis procedure is given below:
A volume V_0' which consists of a volume V_0 of the original serum sample, with total hormone concentration H_0, diluted to V_0' (inclu-

ding the possibility that $V_0 = V_0'$) is dialysed against a volume V_d of buffer. $\underline{C}$ cpm of tracer are added to the system. According to its definition, $c_0 = \underline{C}/V_0$. When equilibrium has been reached, the tracer concentrations in dialysand and dialysate have become c_1 and c_2 while the volumes of these compartments have become V_1 and V_2 respectively. Because of possible fluid shift resulting from osmotic pressure, $V_1 \geq V_0'$ and $V_2 \leq V_d$. So, if required, V_1 and V_2 must be measured. Since the total solution volume of the system (V_t) has not changed it is sufficient to measure V_0'/V_1. The total hormone concentration in the system is H_0V_0/V_t.

By means of these variables the purpose is now to find the best approximation of the free hormone fraction in the original sample, f_0, so that the free hormone concentration for the original, undiluted state can be calculated from $F_0 = f_0 \times H_0$. The free hormone fraction f corresponding to a dilution factor of V_t/V_0 can be obtained by dividing the (free) hormone concentration in the dialysate, F, by the total hormone concentration of the system, i.e. H_0V_0/V_t. When these concentrations of unlabelled moieties are replaced by their radioactive counterparts, i.e. F replaced by c_2 and H_0 replaced by c_0, exactly the same f will result: since labelled and unlabelled species are directly connected by means of the specific activity, and equal specific activity of free and bound was assumed, the ratios of free to total hormone are equal for labelled and unlabelled hormone:

$$\frac{c_2}{c_0V_0/V_t} = \frac{F}{H_0V_0/V_t} = f \qquad \text{(IV-3)}$$

$$= c_2V_2/c_0V_0$$

Of this variable f a lot is known by now. It was already shown that from f a straightforward estimate of f_0 can be obtained using eq. (I-16) which will yield the true value of f_0, if $K_1 + F$ is virtually constant for all K_1 when diluting from V_0 to V_t. This condition was shown in ch.I.4 to correspond to direct proportionality of dilution

and f/b ("linear dilution effect"), which holds more generally than proportionality with f.

When (I-16) and (IV-3) are combined, one obtains f_0' expressed in experimentally assessible variables:

$$f_0' = ((c_0/c_2) - (V_t/V_0) + 1)^{-1} \qquad \text{(IV-4)}$$

This shows that f_0' is readily assessible just from measurements of radioactivity in an aliquot from the dialysate and the tracer solution added. The volumina V_0 and V_t are known and will not change during dialysis.

The above approach to the estimation of the free hormone fraction from equilibrium dialysis data, as formulated by eq.(IV-4) is a logical consequence of the theoretical analysis of ch.I. When comparing it with other approaches as have appeared in the literature, three features must be considered as essential. First, by this method an approximative value of f_0 is calculated from readily available radioactivity measurements (c_0, c_2) which are independent of volume changes during the dialysis process. Secondly, the correction for dilution implicit in the calculation is based on the assumption of direct proportionality between the f/b ratio and dilution (linear dilution effect). Thirdly, the correction for dilution applied, comprises the complete sequence ($V_t \rightarrow V_1 \rightarrow V_0' \rightarrow V_0$) from the sample dilution at dialysis equilibrium back to the original sample by taking into account both the dilution inherent in the dialysis process itself and dilution prior to dialysis, if applied.

Below a number of approaches to free hormone fraction assessment with brief descriptions are presented. Three of these (IV-3, IV-4 and IV-9) originate from this study whereas the other four approaches are taken from the literature but are expressed in the symbols of the present study. The listed methods may differ in several respects: the assay variables they employ, the way dilution is corrected for or the dilution they correspond to.

c_2V_t/c_0V_0; (IV-3), derived in this paragraph.
Free hormone fraction corresponding to (V_t/V_0) times diluted sample, based on assessment of dialysate radioactivity and amount of tracer added.

$((c_0/c_2) - (V_t/V_0) + 1)^{-1}$; (IV-4), derived in this paragraph.
Estimate of free hormone fraction for the undiluted sample, based on assessment of dialysate radioactivity and amount of tracer added in the (V_t/V_0) times diluted state and on the assumption of direct proportionality of f/b and V_t/V_0.

$c_2V_t/(c_1V_1 + c_2V_2)$; (IV-5), equivalent to Oppenheimer et al. (1963, p.1770 loc.cit.).
Free hormone fraction corresponding to (V_t/V_0) times diluted sample, based on assessment of dialysate and dialysand radioactivity and, if necessary, of V_1 and V_2.

$c_2V_0/(c_1V_1 + c_2V_2)$; (IV-6), equivalent to Oppenheimer et al. (1964, eq.(11) loc.cit.).
Estimate of free hormone fraction for undiluted serum obtained by division of (IV-5) by V_t/V_0, thus assuming a direct proportionality between f and V_t/V_0.

c_2/c_0; (IV-7), equivalent to Sterling et al. (1966).
Estimate of free hormone fraction for undiluted serum, based on assessment of dialysate radioactivity and tracer added in the V_t/V_0 times diluted state. It may be considered as the product of (IV-3) and V_0/V_t and as such it is based on the assumption of direct proportionality between f and V_t/V_0.

c_2/c_1; (IV-8), equivalent to Slaunwhite et al. (1959).
Free hormone fraction in the dialysand, based on measurement of dialysate and dialysand radioactivities. If the dialysand volume remains constant during dialysis, (IV-8) gives the free hormone fraction in the dialysand before dialysis, if the same conditions that lead to direct proportionality between f/b and dilution are fulfilled.

$((c_1/c_2) - 1)(V_1/V_0) + 1)^{-1}$; (IV-9), this paragraph (vide infra)
Estimate of free hormone fraction for undiluted sample based on assessment of dialysate and dialysand radioactivities in the (V_t/V_0) times diluted state, assuming a direct proportionality between f/b and dilution.

The free hormone fraction corresponding to a dilution factor of V_t/V_0 (eq.IV-3) is theoretically equal to the "dialysable fraction of serum T4", DF, as given by Oppenheimer (1963). However, DF was not determined from the same experimental data:

$$DF = \frac{c_2(V_{in} + V_{out})}{c_1V_{in} + c_2V_{out}}$$

The symbols used by Oppenheimer for the concentrations of radioactivity inside and outside the dialysis bag at equilibrium can be replaced by c_1 and c_2 respectively. Since in Oppenheimers case the serum was placed inside the bag in 1 : 25 diluted form, it was justified to assume and proven that no fluid shift occurred during dialysis so that in this case $V_{in} = V_0' = V_1$ and $V_{out} = V_d = V_2$. Indeed, in Oppenheimer's article for V_{in} and V_{out} the inside and outside volumes prior to dialysis, i.e. V_0' and V_d were taken. Should one wish to apply the Oppenheimer formula in the general case, it should be read as (IV-5), because generally $V_0' \neq V_1$ and $V_d \neq V_2$. Since $c_1V_1 + c_2V_2$ represents the total amount of tracer measured in the system and c_0V_0 is the total amount added to the system, these quantities should be equal and expressions (IV-3) and (IV-5) would be identical from a theoretical point of view. Experimentally however they are distinct since they employ different sets of data which may lead to actual differences in their result in the case of loss of tracer to cell surfaces or membrane due to adsorption. Since for application of eq.(IV-5) only the tracer which is actually present in solution (c_1, c_2) is assessed, it may be applied regardless of tracer recovery. On the other hand, it necessitates measurement of V_1 and V_2 unless the serum is sufficiently diluted before dialysis. In contrast, the use of eq.(IV-3) obviates the need for measuring V_1 and V_2 but it may be applied only when $c_0V_0 = c_1V_1 + c_2V_2$, i.e. when tracer is quantitatively recovered.

The next step by Oppenheimer is to estimate the concentration of free T4 in the dialysis system, by multiplication of "DF" (eq.IV-5) by the Protein-Bound Iodine (PBI) as a measure for total T4 and the factor V_0/V_t to correct the latter for dilution. In a later

report (Oppenheimer et al. 1964) it is demonstrated from theoretical and practical analysis that, broadly speaking, even for the dilution applied (V_0 = 0.2 ml, V_t = 30 ml, V_t/V_0 = 150), free T4 (concentration) in the diluted state equals that in the diluted state: DF/D = T_d/T, where D = dilution factor, T_d and T are free T4 in the dialysis system (diluted state and the undiluted state respectively) (eq.11 loc.cit.). In terms of the present study it means that the free hormone fraction f, measured and calculated according to (IV-3) or (IV-5) may be corrected for dilution directly by the dilution factor V_t/V_0 assuming a direct proportionality between f and V_t/V_0. Thus, Oppenheimers eq.11 (1964) becomes identical to (IV-6) when inserting (IV-5) and using the symbols of the present study.

The well-known study of Sterling and Brenner (1966) reports dialysis of 3 ml of undiluted serum against 5 ml of buffer. The authors consider the ratio c_2/c_0 as the free hormone fraction corresponding to the undiluted serum, thus disregarding the dilution inherent in the dialysis process itself (V_t/V_0 = 8/3), on which c_2 depends. The same ratio c_2/c_0 can also be obtained by multiplying f according to (IV-3) by V_0/V_t, which shows that the c_2/c_0 ratio in fact is an estimate of the free hormone fraction in the undiluted state, based on measurement in the diluted state (V_t/V_0 = 8/3 in Sterling's case) and corrected by assuming a direct proportionality between f and V_t/V_0, in the same way as (IV-6) was obtained from (IV-5). As both instances apply to T4 in serum the correction for dilution employed finds justification in Oppenheimer's theoretical analysis (1964) and the findings in ch.I,appendix IA.2.2.

For the assay of free steroid hormones, most often the free hormone fraction according to Slaunwhite and Sandberg (1959) is assessed. This procedure consists of measurement of c_1 and c_2 and the ratio $f_s = c_2/c_1$. Since c_1 is the total tracer concentration in the dialysand and c_2 is the concentration of free tracer both in dialysate and dialysand, the ratio c_2/c_1 is equal to the free hormone fraction in the dialysand. It is generally assumed that this corresponds to the free hormone fraction in the dialysand just before dialysis, if

no dialysand dilution caused by fluid shift would occur. Thus, the changes induced in the hormone-protein equilibrium by the dialysis process itself are neglected. Accordingly, f_s is assumed to be identical to f_0 if undiluted serum is dialysed and to f corresponding to a dilution of V_0'/V_0 if a diluted sample is assayed.
The limited validity of these assumptions will become apparent when f_s is expressed as a function of the free hormone concentration F.
Since it is assumed that tracer and unlabelled hormone are equally distributed over free and bound forms, the ratio of dialysate and dialysand radioactive concentrations is identical to the corresponding ratio of unlabelled hormone: $f_s = c_2/c_1 = F/H_1$.
The hormone (bound + free) concentration H_1 in the dialysand was given in ch.I.5 by eq.(I-20). In that paragraph it was demonstrated that F' in (I-20) may be replaced by F so that here it is possible to express $f_s = c_2/c_1 = F/H_1$ in terms of the free hormone concentration F and binding parameters:

$$f_s = ((V_0/V_1)\ \Sigma\ \frac{P_{i0}}{K_i + F} + 1)^{-1} \qquad \text{(IV-10)}$$

The difference between f_0, f and f_s can be demonstrated by comparing (IV-10) with eqs.(I-15a) and (I-15b):

$$f = ((V_0/V_t)\ \Sigma\ \frac{P_{i0}}{K_i + F} + 1)^{-1} \qquad \text{(I-15a)}$$

$$f_0 = (\Sigma\ \frac{P_{i0}}{K_i + F_0} + 1)^{-1} \qquad \text{(I-15b)}$$

Comparison of (IV-19) with (I-15b) shows that, even if $V_1 = V_0$, which means that undiluted serum is dialysed and no fluid shift occurs, in general $f_s \neq f_0$ because of the appearance in (IV-10) of F instead of F_0. Only in the case that, in addition to $V_1 = V_0$, also $K_i + F = K_i + F_0$ (a well-known condition), eqs.(IV-10) and (I-15b) become identical and $f_s = f_0$. By comparison of (IV-10) and (I-15a) it is easily understood that f_s in practice never equals f, since $V_t \neq V_0$.

A relation between f and f_s can be derived by eliminating $\Sigma P_{i0}/(K_1 + F)$ from (I-15a) and (IV-10]:

$$f_s = ((V_t/V_1)(1/f - 1) + 1)^{-1} \qquad \text{(IV-11)}$$

For the case that again $V_1 = V_0$, this expression is identical to (I-16):

$$f_0' = ((V_t/V_0)(1/f - 1) + 1)^{-1} \qquad \text{(I-16)}$$

So that $f_s = f_0'$. The conditions for which $f_0' = f_0$ have already been discussed in ch.I.4. Obviously these are identical to those leading to equality of (IV-10) and (I-15b) (vide supra). While f_0' is an estimate of the free hormone fraction if the sample would occupy a volume V_0, f_s is an estimate computed in the same way of the free hormone fraction corresponding to dilution of the sample to a volume V_1. In order to obtain f_0' from f_s, in the general case that $V_0 \neq V_1$, the following expression, derived by elimination of f from (I-16) and (IV-11) must be used:

$$f_0' = ((V_1/V_0)(1/f_s - 1) + 1)^{-1} \qquad \text{(IV-12)}$$

in experimental variables expressed as:

$$f_0' = (V_1/V_0)(c_1/c_2 - 1) + 1)^{-1} \qquad \text{(IV-9)}$$

This demonstrates that when using the c_2/c_1 ratio to estimate f_0', always the dilution in the dialysand has to be taken into account, whether it arises from dilution prior to dialysis or from fluid shift during the process. Thus in may instances it requires measurement of volume changes. However, (IV-9) may have an advantage over (IV-4) which estimates f_0' from c_2, c_0, V_0 and V_t, since the latter employs added tracer (c_0) for the calculation whereas (IV-9) is based on tracer concentrations actually present in the system (c_2, c_1). Analogous to the case of (IV-3) vs. (IV-5), eq.(IV-9) may always be applied, in particular when some tracer is lost by adsorption, but requires measurement of V_1/V_0, whereas (IV-4) may be

applied only when tracer is quantitatively recovered. This requires prior examination of tracer recovery in a pilot experiment by comparing c_0V_0 with $c_1V_1 + c_2V_2$ using the following formula:

$$\text{rec.} = \frac{c_1V_1 + c_2(V_t - V_1)}{c_0V_0} \cdot 100\% \qquad \text{(IV-13)}$$

The significance of eqs.(IV-3) - (IV-9) can be further illustrated by their application to the following data which were taken from the experiment in IV.4.1 on free cortisol.

c_1 = 14589 cpm/ml
c_2 = 1213 "
c_0 = 18158 "
V_0 = 0.75 ml
V_t = 2.00 "
V_1 = 0.84 "
V_2 = 1.16 "

The following results are obtained:

(IV-3)	0.178
(IV-4)	0.0752
(IV-5)	0.178
(IV-6)	0.0666
(IV-7)	0.0668
(IV-8)	0.0831
(IV-9)	0.0749
(IV-13)	100.3%

These results confirm that (IV-3) and (IV-5) give the free hormone fraction corresponding to the diluted state. Expressions (IV-6) and (IV-7) give corrected values based on the assumption of proportionality of f and dilution, whereas (IV-4) and (IV-9) employ the

assumption of the linear dilution effect on f/b. The higher value found by means of (IV-8) reflects in fact that with Slaunwhites approach an estimate of the free hormone fraction corresponding to the prevailing dialysand dilution is made (see (IV-11)). The dialysand dilution V_1/V_0 in this case is 1.12. This factor may serve to correct the result according to (IV-12), which obviously yields the same value as direct application of (IV-9).

At this stage it has become possible to judge which evaluation methods are the most useful ones. For measurement of the true free hormone fraction f belonging to a certain dilution, always eq.(IV-5) can be used. It necessitates however measurement of V_1 and V_2. Obviously measurement of V_1/V_0 will also serve the purpose. If the added tracer is quantitatively recovered, which can be verified by means of eq.(IV-13) also eq.(IV-3) may be applied so that measurement of V_1 and V_2 is not necessary. The same holds for the estimation of f_0'. Eq.(IV-9) may always be applied but requires measurement of dialysand dilution, whereas (IV-4), which obviates the need for such measurement, may be applied only when tracer is quantitatively recovered.

If f and f_0' are multiplied by H_0V_0/V_t and H_0 respectively, F and F_0' are obtained. With these variables assessed, one should proceed according to the guidelines for the direct method given in the previous paragraph. The above approach to the assessment of free hormone by means of the indirect equilibrium dialysis method and the concomitant discussion of existing methods was based on the conclusion of ch.I.5 on the equivalence of equilibrium dialysis and dilution, which implies that the free hormone concentration depends only on the ratio of the volume V_0 of original sample present in the system and the total volume V_t of the system.

Some aspects or consequences of this observation are amenable for experimental substantiation, whereas others are difficult to evaluate. One aspect that is particularly suited for experimental verification is the independence of free hormone concentration on dialysand dilution (V_1/V_0) which is actually carried out in IV.3.1 and IV.4.1 for free aldosterone and free cortisol respectively. In the same experiment it is also verified that determinations of f_0', irrespective whether (IV-4) or (IV-9) are used, are independent of

dialysand dilution. True sample dilution V_t/V_0 obviously has a marked effect which is shown in IV.3.2 and IV.4.2. The first describes an example of a linear dilution effect whereas the second (free cortisol) describes a situation where no simple relations for the effect of dilution can be found. The possibility of determining the free hormone fraction corresponding to the undiluted state if limiting conditions hold and the intrinsic impossibility of such a determination without these conditions also can be experimentally assessed by means of comparison of true free fractions obtained from dialysing samples in concentrated form and using (IV-3), with free fractions obtained by dialysing the samples themselves and estimating the free fraction according to (IV-4). Aldosterone and cortisol were chosen for the experiments so that both an instance of a simple relation between free hormone and dilution and an example of a hormone-multiple binding site system in which no simplifying conditions hold can be studied. Moreover, with these hormones possible artefacts due to tracer contamination can be relatively easily managed, such in contrast with the thyroid hormones.

IV.3. Free aldosterone assay by equilibrium dialysis.

IV.3.1. The effect of dialysand dilution.

In 0.2 ml cells 0.175 ml of a serum pool, to which ^3H-Aldosterone tracer had been added, was dialysed against 0.175 ml of phosphate buffer (series A) or 0.175 ml of the same sample was dialysed together with 0.025 ml of buffer against 0.150 ml of buffer (series B). Thus, V_t/V_0 for both series (fivefold determinations) was 2 and the dialysand dilutions prior to dialysis (V_0'/V_0) were 1 and 1.143 respectively.
After 4 hrs dialysis at 37°C, 0.1 ml aliquots of dialysate and dialysand were counted for radioactivity (4 minutes), simultaneously with 0.1 ml aliquots of the original sample. Thus, after due correction for quenching the variables c_2, c_1 and c_0 were obtained.

In addition, in 0.010 ml aliquots from dialysand and original sample total protein determinations were carried out (Folin-Ciocalteu method) for estimation of dialysand dilutions V_1/V_0 at equilibrium. Tracer purity was checked by paper chromatography according to de Man et al. (1977) and found to be better than 98%, which was assumed to be adequate for this specific experiment.
Thus, the complete set of relevant data (c_0, c_1, c_2, V_1/V_0) is obtained (see table IV.1).
First the theoretical conclusion of I.5 that dialysand dilution (V_1/V_0) does not influence the free hormone concentration, as long as the "true" or "total" dilution (V_t/V_0) is constant, is verified. Since from (IV-3) follows that $c_2 = Fc_0/H_0$, where c_0 and H_0 are constants, it is sufficient to demonstrate that c_2 is independent of dialysand dilution in order to prove the same for F. This is done by statistical comparison of the means of measured c_2 values for the two different V_1/V_0 ratio's. For series A (initial dialysand dilution $V_0'/V_0 = 1$) the dialysand dilution at equilibrium, V_1/V_0 was 1.112 $\pm$ 0.0471, and the mean $\pm$ S.D. of dialysate radioactivity c_2 was 2479 $\pm$ 67 cpm/ml. For series B (V_0'/V_0) = 1.143) V_1/V_0 was 1.201 $\pm$ 0.0295 and c_2 was 2468 $\pm$ 37 cpm/ml. No statistical difference was detected for c_2 between series A and B (Student's t test). The pooled estimate of the S.E.M. was 24 cpm/ml, i.e. 0.97%. The 95% confidence interval for the difference between the means had a width of 2x 79 cpm/ml which means that real absolute differences of means exceeding 79 cpm/ml (i.e. 3.18%) would have been detected by this experiment.

With data from the same experiment, f_0' values were calculated for both dialysand dilutions (series A and B) using both calculation methods for f_0', i.e. eq.(IV-4) and eq.(IV-9). These calculations required the additional data on c_0 and c_1. After correction for quenching (efficiency ratio serum/buffer 1.066) c_0 was found to be 8430 $\pm$ 78 cpm/ml, c_1 (A) was 5719 $\pm$ 76 cpm/ml and for c_1 (B) it was 5342 $\pm$ 89 cpm/ml (means $\pm$ S.D.). The difference in c_1 values reflects the difference in dialysand dilution both prior to dialysis and amplified by fluid shift as a consequence of osmotic pressure during the process. The dialysand dilution originating from the

Sample	Dialysand counts/4' per 0.1 ml	Dialysate counts/4' per 0.1 ml	Dialysand dilution initial V_0'/V_0	final V_1/V_0	c_0 = 8430 cpm/ml c_1 cpm/ml	c_2 cpm/ml
A	24752	9500	1.00	1.164	5805	2375
	24563	10037		1.056	5761	2509
	24427	10054		1.159	5729	2513
	23888	10185		1.092	5602	2546
	24303	9811		1.092	5699	2453
B	23327	9635	1.143	1.212	5471	2409
	22982	9944		1.203	5390	2486
	22690	9853		1 241	5321	2463
	22380	9900		1.186	5248	2475
	22526	10025		1.161	5283	2506

Table IV 1
Individual values for radioactivity counts in dialysand and dialysate, initial and final dilution factors, obtained by dialysis of 0.175 ml samples of ^{3}H aldosterone containing poolserum against 0.175 ml phosphate buffer (samples A) or by dialysis of 0.175 ml serum + 0.025 ml buffer against 0.150 ml buffer (samples B).

latter phenomenon only, is readily calculated from V_0'/V_0 and V_1/V_0 values. For series A fluid shift accounted for the complete 11.2% dilution that occurred during the process while for series B it amounted to 5.1%. As shown in table IV.2, values of f_0' (expressed in %) calculated by means of (IV-4) which employs only c_2, c_0 and V_t/V_0 did not differ for the two dialysand dilutions; obviously as a consequence of the independence of c_2 on V_1/V_0. However, in conformity with the theory, also no effect of dialysand dilution was detected when eq.(IV-9), which does employ c_1 and V_1/V_0, was used. Additionally, the results of both calculation methods did not seem to differ either. In order to use the available data on f_0' as efficient as possible and to avoid carrying out 6 pairwise tests, a two-way analysis of variance (ANOVA) was carried out

	c_1 cpm/ml	c_2 cpm/ml	V_1/V_0 initial	final	f_0'(IV-4) (%)	f_0'(IV-9) (%)	rec. (%)
A	5719	2479	1.00	1.112	41.68	40.75	101.7
n=5	± 76	± 67		± 0.0471	± 1.592	± 1.601	± 0.65
B	5342	2468	1.143	1.201	41.39	41.70	99 5
n=5	± 89	± 37		± 0.0295	± 0.867	± 1 307	± 1.05

Table IV 2

Further elaboration of the data from table IV.1. on free aldosterone assay. Means ± S.D. of c_1, c_2, V_1/V_0, recovery (according to eq.IV-13) and estimates f_0' of the free hormone fraction in undiluted serum f_0 computed from c_2, c_0 and V_t/V_0 ("true" dilution) by means of eq.(IV-4) and from c_1, c_2, V_1/V_0 (dialysand dilution) and V_t/V_0 (eq.IV-9).

on the f_0' data obtained by two different calculation methods on two different dialysand dilutions. This statistical analysis would disclose whether one or both factors (calculation method and dialysand dilution) contributes significantly to the overall variability of the observations or that this variation can be explained from replication error only. In this experiment, the latter appeared to be the case i.e. no effect of calculation method or dialysand dilution could be detected. As the error due to replication only (experimental or residual error) was 1.373% (i.e. a residual coefficient of variation of 3.32%), the pooled S.E.M. was 0.614%, that is 1.48% of the overall mean (41.380%) which gives an indication of the precision of the assay. The 95% confidence interval for the difference between any two means was found to have a width of 2x 1.842%, so that this experiment must have been capable to detect absolute real differences between means of 1.842%, i.e. 4.45% of the overall mean f_0'.
In fact, the equality of f_0' values obtained by both calculation methods is ample proof that the tracer recovery must have been very near 100%. For completeness, recoveries were calculated for all ten samples using (IV-13) (see table IV.2).

IV.3.2. The effect of true sample dilution.

A sample of pooled plasma with a total aldosterone level (H_0) of 61 pmol/l was diluted 1.25, 1.50, 1.75, 2.0, 2.5, 3.0, 4.0 and 5.0 - fold and 0.190 ml of these dilutions and of the original sample were dialysed in duplicate against equal volumes of buffer containing ^{3}H-aldosterone tracer. The volumes of undiluted sample actually assayed (V_0) are found from: V_0 = 0.190 ml/(dilution factor V_0'/V_0), so that V_0 becomes 0.190, 0.152, 0.127 etc. ml for dilution factors V_0'/V_0 of 1, 1.25, 1.5 etc. Since V_t = 2 x 0.190 ml = 0.380 ml the values of V_t/V_0 were 2, 2.5, 3, etc. (see table IV.3). After equilibrium had been reached, 0.1 ml aliquots of dialysates were counted for 10'. Tracer contamination was assessed by dialysis of an antiserum against aldosterone to which tracer was added, against an equal volume of buffer. As a consequence of the high affinity of the antibody for aldosterone it may be assumed that initially all intact ^{3}H-aldosterone binds to the antibody and thus remains in the antiserum compartment. In contrast, unbound radioactive compounds will be distributed over the complete volume of the dialysis system. If $\underline{C}$ cpm of radioactive material was added and a concentration of ε cpm/ml is found in the dialysate, then the contamination amounts to $\varepsilon.V_t/\underline{C}$ x 100% (V_t expressed in ml). The tracer used for this experiment was found to be contaminated with 1.17% radioactivity that does not bind to antibody. In total, 42426 counts/10' were added to each cell, of which 100 - 1.17% = 4193 cpm was considered as intact ^{3}H-aldosterone, so c_0V_0 = 4193 cpm. The values of c_0 for the various dilutions were found by division of this 4193 cpm by V_0. The remaining 1.17% contamination, which is 50 cpm of the amount of tracer added is distributed over the total volume of the dialysis system (0.380 ml) so it contributes 130 cpm/ml to the concentration of radioactivity both in dialysate and dialysand, so that in order to obtain accurate values for c_2 and c_1, the 130 cpm/ml must be substracted from measured radioactivity. In table IV.3 a complete account of V_0'/V_0, V_t/V_0 and V_0 values are given, together with c_0 and c_2 values that were corrected for tracer contamination.
From these data the values of f corresponding to each dilution

V_0'/V_0	V_t/V_0	V_0	c_0 cpm/ml	c_2(individual) cpm/ml	c_2(mean) cpm/ml
1	2	0.190	22068	7138	7112
				7085	
1.25	2.5	0.152	27586	7418	7319
				7220	
1.50	3.0	0.127	33016	7931	7992
				8053	
1.75	3.5	0.109	38478	8306	8398
				8489	
2.0	4.0	0.0950	44137	8426	8426
2.5	5.0	0.0760	55171	9154	8834
				8513	
3.0	6.0	0.0633	66240	9176	9176
4.0	8.0	0.0475	88274	9633	9630
5.0	10.0	0.0380	110342	9827	9827
				9827	

Table IV.3.

Dialysis of different dilutions (V_0'/V_0) of poolplasma against an equal volume of buffer, to which ^{3}H-aldosterone was added. Given are "true" dilution factors (V_t/V_0), the volume of undiluted, original plasma actually assayed (V_0), the concentration of radioactivity corresponding to the original sample (c_0) and in the dialysates at equilibrium.

V_t/V_0 were calculated with eq.(IV-3). Subsequently $F = f.H_0V_0/V_t$ and $f/b = f/(1-f)$ were calculated (table IV.4) and plotted against V_t/V_0 (fig.IV.1). Also f_0' was calculated for each dilution by means of (IV-4) (table IV.4).

A linear dilution effect was strongly indicated by the finding that f_0' values did not differ, and that no correlation between f_0' and V_t/V_0 was found ($r = -0.031$). When the dilution effect is approached as a relation between f/b and dilution a highly significant correlation is found ($r = 0.9971$) of which even the lower 95% confidence limit exceeds $r = 0.99$.

The free aldosterone concentration F is shown to fall progressively upon dilution so that it is concluded that the observed linearity of the dilution effect in this instance is a consequence of $F_0 << K_i$ ($i = 1 \ldots\ldots n$), as was already anticipated (appendix IA.2.1.). The constancy of f_0' in fact provides ample evidence that in this case it is a valid estimator of f_0. For the purpose of illustration however, "true" f_0 values, which can be obtained for diluted serum, are compared with the corresponding f_0' values. All values of f calculated by means of eq.(IV-3) are in fact f_0 values when V_t/V_0 times diluted serum is considered as the original sample. So, when 1.25 times diluted serum is dialysed against an equal volume of buffer, $V_t/V_0 = 2.5$ and the free hormone fraction f found by applying (IV-3) is equal to f_0 for a sample consisting of 2.5 times diluted serum. When 2.5 times diluted serum is dialysed against an equal volume of buffer, the f_0 for this sample (denoted by $S_{2.5}$) can be approximated by determining f_0' by means of eq.(IV-4), taking $V_t/V_0 = 2$, instead of 5, since V_0 now corresponds to $S_{2.5}$ instead of S_1 (undiluted). The above may be further illustrated by substituting real data. Table IV.4 shows that dialysis of 1.25 times diluted serum ($V_0'/V_0 = 1\ 25$, $V_t/V_0 = 2.5$) gives $f = 0.663$ which, according to the above is equal to f_0 for $S_{2.5}$. Dialysis of 2.5 times diluted serum ($V_0'/V_0 = 2.5$, $V_t/V_0 = 5$) gives $c_2 = 8834$. Now, since in total 4193 cpm of tracer were added, and V_0 for a 2.5 times diluted sample is 0.190 ml, $c_0 = 22068$ cpm/ml, and $V_t/V_0 = 2$. When these values are substituted in (IV-4) one obtains $f_0' = 0.668$. A number of such comparisons are presented in table IV.5.

V_t/V_0	f(IV-3)	f_0'(IV-4)	f/b	F(pmol/l)
2	0.644	0.476	1.81	19.7
2.5	0.663	0.441	1.97	16.2
3	0.724	0.467	2.63	14.7
3.5	0.761	0.476	3.19	13.3
4*	0.764	0.447	3.23	11.6
5	0.801	0.445	4.02	9.77
6*	0.832	0.452	4.94	8.45
8*	0.873	0.462	6.88	6.66
10	0.891	0.449	8.14	5.43

Table IV 4

Further elaboration of the data from table IV.3. on free aldosterone assay of serially diluted plasma for evaluation of the effect of dilution on the true free hormone fraction f, the estimate f_0' that can be calculated of the free hormone fraction corresponding to the undiluted state, the ratio of free to bound hormone (f/b) and the actual concentration of free aldosterone (F).

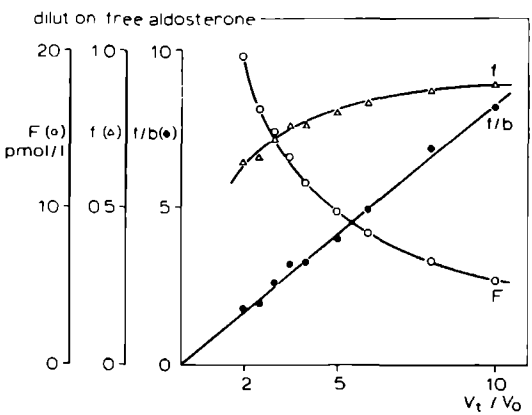


Fig.IV.1.

The effect of dilution on free aldosterone in a normal pool-plasma as determined by equilibrium dialysis. The data were taken from table IV.4. The linearity of the f/b curve demonstrates the possibility of correcting for dilution using eq.(IV-4) to obtain an accurate estimate of f_0.

	f_0 (IV-3)	$f_{(0')}$ (IV-4)
S_2	0.6445	0.6176
$S_{2.5}$	0.6633	0.6675
S_3	0.7243	0.7118
S_4	0.7636	0.7747
S_5	0.8006	0.8028

Table IV 5

"True" (f_0) and estimated (f_0') free aldosterone fractions of diluted serum samples, obtained by dialysing the twice concentrated sample and the sample itself, respectively, against an equal volume of buffer (for further comment see text).

IV.4. Free cortisol assay by equilibrium dialysis.

The purpose of the experiments described below is largely the same as in the previous paragraph on free aldosterone. However, since free hormone fractions in the case of cortisol are considerably lower than in the case of aldosterone, some points (tracer contamination, effect of dialysand dilution on counting efficiency) are considered and assessed in more detail as they are expected to have a larger effect on the results.

IV.4.1. The effect of dialysand dilution.

The first experiment consisted of dialysis in triplicate of 0.75 ml serum in the presence of ^{3}H-cortisol at three different dialysand dilutions (V_1/V_0) but constant total dilutions (V_t/V_0) in 1 ml cells as follows:

0.75 ml poolserum vs. 1.25 ml buffer (series A)
0.75 ml " + 0.25 ml buffer vs. 1.0 ml " (series B)
0.75 ml " + 0.5 ml " vs. 0.75 ml " (series C)

Thus, the initial dialysand dilutions (V_0'/V_0) were 1, 1.333 and 1.667 respectively, whereas the total (true) dilution V_t/V_0 was 2.667 in all three cases.

At the beginning, 10 µl of a ^{3}H-cortisol solution was added to each cell. After dialysis for about 7 hrs, 0.5 ml aliquots of the dialysates and 0.1 ml aliquots of dialysands were counted for 10 minutes. Dialysand dilution V_1/V_0 was assessed by total protein determination: Series A: 1.125, B: 1.389 and C: 1.720. In order to determine c_0 and to assess the proportions of counting efficiencies for the different compositions of aliquots counted, the following solutions were prepared for radioactivity counting in quadruplicate:

10 µl tracer + 0.75 ml serum	counted: 0.1 ml	(T_S)
10 µl " + 0.75 ml buffer	" : 0.1 ml	(T_{B1})
10 µl " + 0.75 ml "	" : 0.5 ml	(T_{B2})

The mean concentrations of radioactivity (cpm/ml) found were: T_S: 18056, T_{B1}: 18430 and T_{B2}: 17501. When the counting efficiency of T_{B1} is taken as unity, the relative counting efficiency for a 0.1 ml undiluted serum sample is 0.9797 and for 0.5 ml buffer - the form in which dialysate is counted - it is 0.9252. The relative counting efficiencies for the dialysands are calculated as: $1 - (1 - 0.9797)V_0/V_1$. This results in relative efficiencies of 0.9820, 0.9854 and 0.9881 for the dialysands of A, B and C. The dialysand counts have to be divided by these factors and the dialysate counts by 0.9252 in order to obtain c_1 and c_2 values that are comparable with c_0. After correction for differences in efficiency, a correction of c_0, c_1 and c_2 values for tracer contamination was applied. It had been found by means of a similar test as outlined in IV.3.2 that the ^{3}H-cortisol consisted for 1.50% of radioactivity that does not bind to excess cortisol antibody. Hence c_0 becomes 18514 cpm/ml and since 0.75 x 18430 cpm of tracer is present in each dialysis cell it contains 0.015 x 0.75 x 18430 = 207 cpm of non-cortisol radioactivity, which is assumed to distribute itself over 2 ml, so that it acquires a concentration of 104 cpm/ml both in dialysate and dialysand. In table IV.6 the gross counts in the 0.1 ml dialysand and 0.5 ml dialysate are given followed by

Sample	Dialysand		Dialysate		Dialysand dilution		c_0 = 18158 cpm/ml		
	counts/10' per 0.1 ml	eff. ratio	counts/10' per 0.5 ml	eff. ratio	initial V_0'/V_0	final V_1/V_0	c_1 cpm/ml	c_2 cpm/ml	rec. %
A	14386	0.9820	5921	0.9252	1.00	1.125	14546	1176	100.11
	14386		6045				14546	1203	100.34
	14514		6310				14676	1260	101.62
B	12293	0.9854	6014	0.9252	1.333	1.389	12371	1196	103.05
	11936		6011				12009	1195	100.27
	12097		6338				12172	1266	102.02
C	10204	0.9881	6130	0.9252	1.667	1.720	10223	1221	103.20
	10123		5894				10141	1170	102.16
	10076		5953				10093	1183	101.77

Table IV 6

Individual values of radioactivity counts obtained by dialysis of 0.75 ml poolserum with 0, 0.25 and 0.5 ml buffer added, against 1.25, 1.00 and 0.75 ml buffer.
^{3}H cortisol was added to the dialysand. Also shown are relative efficiencies and initial and final dialysand dilution factors.
Values for c_0, c_1 and c_2 are corrected for efficiency differences and tracer contamination and recoveries are calculated according to eq.(IV-13).

	c_1 cpm/0.1 ml	c_2 cpm/0.1 ml	V_1/V_0 initial	final	f_0' (IV-4) (%)	f_0' (IV-9) (%)	rec. (%)
A	14589	1213	1.00	1.125	7.518	7.480	100.7
n=3	± 75	± 43			± 0.300	± 0 265	± 0.81
B	12184	1219	1.333	1.389	7.560	7.431	101.2
	± 181	± 41			± 0.285	± 0 313	± 1.62
C	10152	1191	1.667	1.720	7.367	7.174	102.4
	± 66	± 27			± 0 184	± 0 131	± 0.74

Table IV 7

Summary of data (means ± S.D.) on free cortisol assay of table IV.6. Also means and standard deviation are given of the estimates of the free hormone fraction for undiluted serum, as calculated from these data by means of (IV-4) and (IV-9).

the corresponding c_1 and c_2 values obtained by correction for efficiency and tracer contamination. Additionally the tracer recovery as calculated by means of (IV-13) is given.

The absence of any effect of dialysand dilution on the tracer concentration c_2 in the dialysate (tables IV.6 and 7) was verified by means of single factor ANOVA. The residual error was 40.6 cpm/ml so that the coefficient of variation of the means of dialysate concentrations was 1.8% and the confidence interval of differences between means had a width of 2 x 81.1 cpm/ml so that absolute differences between any two means exceeding 6.7% (expressed as percentage of overall mean c_2) would have been detected by this experiment as significant. From the data on c_0, c_1, c_2, V_1/V_0 and V_t/V_0, f_0' values were calculated for each dialysand dilution both by eq.(IV-1) and (IV-9). The results are summarized in table IV.7. Two factor ANOVA on the six mean f_0' values showed no significant difference between any two means. The residual error was 0.255% (expressed in percentage free) i.e. 3.4% of the overall mean f_0' (7.422%). The width of the 95% confidence interval was 2 x 0.458%, i.e. 6.2% of the overall mean.

IV.4.2. The effect of the true sample dilution.

For assessment of the effect of true dilution (V_t/V_0) on free cortisol the direct method for free hormone assay was used. 0.150 ml samples of a series of solutions consisting of undiluted, 2-, 4-, 8- and 16-fold diluted poolserum were dialysed in quadruplicate against equal volumes of buffer. At equilibrium (about 4 hrs) 0.1 ml aliquots of the dialysate were taken for cortisol radioimmunoassay and the results expressed in nmol/l. Also in the original, undiluted serum cortisol was determined. In this way in fact F at different dilutions and H_0 were measured, but these can be substituted for c_2 and c_0 in eqs.(IV-3)-(IV-13), if required. In table IV.8 the initial dilutions V_0'/V_0, the true dilutions V_t/V_0, V_0, c_0 (= H_0) and c_2 (= F) are given for the different samples.
The effect of dilution V_t/V_0 on F was assessed directly, the effect

V_0'/V_0	V_t/V_0	V_0 ml	$H_0(c_0)$ nmol/l	$F(c_2)$ nmol/l	
1	2	0.150	582.5	62.4	54.9
				54.2	46.8
2	4	0.075	582.5	39.4	39.4
				38.6	45.0
4	8	0.9375	582.5	28.2	31.2
				31.9	31.9
8	16	0.01875	582.5	18.6	20.8
				18.6	17.8
16	32	0.009375	582.5	11.9	10.4
				10.4	10.4

Table IV 8

Assay of free cortisol by direct radioimmunoassay in equilibrium dialysates of undiluted, 2-, 4-, 8- and 16-fold diluted poolserum. Dialysis was carried out in duplicate as well as radioimmunoassay. The results (values of F) belonging to the same dialysate appear below each other. For H_0 the following individual values were found: 610, 580, 610, 530 nmol/l.

on f after calculation of f by means of (IV-3), and f_0' is calculated for the different dilutions by (IV-4). F_0' can be assessed either by multiplication of f_0' by H_0 or by direct calculation from F, V_t/V_0 and H_0 by means of eq.(I-14), which methods are equivalent.

The results of measurements of F and computation of f/b and f are given in table IV.9 and are plotted against V_t/V_0 in fig.IV.2. Whereas in the case of free aldosterone a linear dilution effect was found, in the present case it was clearly not so. This is reflected by the fact that f_0' estimates (table IV.9) are not constant. To exclude the possibility that some of the differences originated from experimental error, all possible combinations of f_0' values were tested using the Newman-Keuls procedure for pairwise comparisons. This procedure safeguards against the risk of obtaining significance by mere chance when carrying out a large number of Student's t-tests. It was found that only the f_0' values obtained from $V_t/V_0 = 4$ and 8 did not differ significantly. In contrast, plots of f and F do not seem to display a pattern essentially different from that of fig.IV.1, so no direct information about the dilution effect is obtained from these relations. Also in this experiment it is possible to compare true f_0 values obtained for diluted serum by dialysis in its concentrated form, with f_0' values obtained from dialysis of the diluted serum as such. The different samples are denoted, according to their initial dilution factor V_0'/V_0 by S_1, S_2, S_4, S_8 and S_{16}. To repeat briefly, f_0', is assessed from dialysis of samples S_2, S_4, S_8 and S_{16} against equal volumes of buffer and calculating by eq.(IV-4) with $V_t/V_0 = 2$ in all cases and $c_0 = H_0/(\text{initial dilution } V_0'/V_0)$, whereas the corresponding f_0 values are equal to the values of f obtained through dialysis of samples S_1, S_2, S_4 and S_8 and calculations according to eq.(IV-3). The results are given in table IV.10. The data in this table were analysed by two-factor ANOVA which demonstrated that the difference of the two assays yielding f_0 and f_0' was highly significant ($p < 0.001$) while also dependence on the sample of the effect of the difference in method (interaction) was discernible ($p < 0.02$).

V_t/V_0	f (IV-3)	f_0'(IV-4)	f/b	F (nmol/l)	F_0'(IV-4) (nmol/l)
2	0.187	0.1032	0.230	54.5	60.1
	± 0.02107	± 0.01278		± 0.61	
4	0.279	0.0882	0.387	40.6	51.3
	± 0.0203	± 0.00829		± 0.306	
8	0.423	0.0842	0.732	30.8	48.9
	± 0.0242	± 0.00746		± 0.18	
16	0.521	0.0640	1.086	18.9	36.8
	± 0.0354	± 0.00891		± 0.13	
32	0.592	0.0439	1.451	10.8	25.3
	± 0.0412	± 0.00786		± 0.75	

Table IV 9

Free cortisol fractions for different dilutions (f), with correction for dilution (f_0'), the free over bound ratio (f/b) and the free cortisol concentration (F) after correction for dilution (F_0') as calculated from the data of table IV.8.

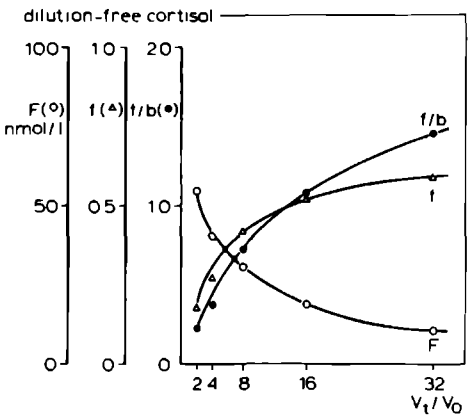


Fig.IV.2.

The effect of dilution on free cortisol in a normal poolserum determined by equilibrium dialysis with radioimmunoassay in the dialysates. Data taken from table IV.9. Non-linearity of all curves demonstrated that it is not possible by any known means to obtain an accurate estimate of the free hormone in the undiluted sample.

Sample	f_0(IV-3)	f_0'(IV-4)
S_2	0.187	0.162
	± 0.0211	± 0.0139
S_4	0.278	0.268
	± 0.0203	± 0.0192
S_8	0.0423	0.352
	± 0.0242	± 0.0330
S_{16}	0.521	0.421
	± 0.0354	± 0.0428

Table IV 10

"True" (f_0) and estimated (f_0') free cortisol fractions of diluted serum samples, obtained by dialysing the twice concentrated sample and the sample itself, respectively, against an equal volume of buffer (for further comment see text).

IV.5. Discussion

This chapter deals mainly with one type of problem in equilibrium dialysis: the different forms of dilution inherent in or applied with the method.
The effects observed and their role in evaluation of measurement data are a reflection of the hormone-protein equilibria involved and as such they are fundamentally connected with the phenomenon of hormone binding to proteins. This contrasts with the other main problem in equilibrium dialysis, as far as the indirect method is concerned, i.e. the effect of tracer contamination, which is purely artefactual in nature. Therefore in the context of this chapter it received less attention. Instead, an analysis of the effect of unbound and weakly bound tracer impurities is simultaneously given for equilibrium dialysis and symmetric dialysis in ch.V.4. This analysis is carried out on the free hormone assay that suffers most from this artefact, viz. the free T4 assay.
Because tracer contamination constitues a major problem in free T4 and T3 assay by equilibrium dialysis, these assays are cumbersome

so the general dilution - dialysis - free hormone relationships can be much better studied by means of assays which are not troubled by this type of interference.

Since discussion of the concepts of handling of equilibrium dialysis data took place simultaneously with their introduction there does not much remain for discussion on this subject. However, two points deserve mentioning. In the literature no alternative to or equivalent of eq.(I-14) is found for estimation of F_0 from direct free hormone assay in equilibrium dialysates. In free thyroid hormone assays by this method the authors (Ekins et al. 1975) are aware of the fact that the free hormone concentration measured corresponds to the actual dilution of the sample in the dialysis system. This concentration would not differ very much from F_0, when dilution effects as described by Spaulding et al. 1971, Pedersen 1974, Olsen 1977 are properly dealt with, so there is no need for dilution correction in that case. A quite different situation exists when free cortisol is assessed by this method, since then F differs appreciably from F_0.

Assay of free cortisol by radioimmunoassay of cortisol in equilibrium dialysate of undiluted serum was described by Clerico et al. (1978). These authors then calculate the absolute amounts of total and free cortisol present in the dialysand, using measurements of dialysand volume change, in order to obtain the free hormone fraction according to Slaunwhite, thus introducing the error caused by dialysand dilution where it was originally absent. Estimation of F_0 by means of eq.(I-14), in contrast, was shown to be independent of dialysand dilution.

It is possible to correct the free hormone fraction in the dialysand (f_s), as assessed according to Slaunwhite, for dialysand dilution by an approach that makes use of the independence of free hormone on dialysand dilution. In this approach the concentration of radioactivity in the dialysand is corrected for the amount of dialysate that has been transferred to the dialysand. The result of such correction can be readily demonstrated to be identical to eq.(IV-9), which was derived by expressing f_s first in terms of the true free hormone fraction f belonging to a dilution V_t/V_0 (eq.

IV-11). The former approach was aimed at by Kley et al. (1977) but unfortunately a flaw crept into their formula.

The remainder of this discussion will be devoted to critical review of the experimental validation of the concepts developed in ch.IV.2. With regard to the effects of dialysand dilution the following remarks are made:

Although not shown in the results, it is easily seen that because of constancy of c_2, free hormone fractions calculated according to Slaunwhite ($f_s = c_2/c_1$) would yield results that are very much dependent on dialysand dilution, because of the significant decrease of c_1. Calculation of f by means of $f = c_2V_t/c_0V_0$ eq.(IV-3) was not shown because of its direct dependence on the constancy of c_2, so that it would not provide any additional information. This is also the case for eq.(IV-5) which differs only from (IV-3) in the way the total radioactivity present is assessed. This is a matter of tracer recovery only, which is presented for each assay series. For the same reason the agreement between the results for f_0' obtained by eqs.(IV-4) and (IV-9) may be viewed merely as the consequence of the virtually quantitative tracer recovery. In fact, the only really independent observations are the constancy of c_2 and the equality of c_0V_0 and $c_1V_1 + c_2V_2$, of which the agreement between f_0' values for both calculation methods and all dialysand dilutions are merely necessary consequences. However, because of the computations involved, it would be hard to predict from observations on c_2 and tracer recovery alone whether a real agreement between all f_0' estimates would exist, and therefore the computed values for f_0' are themselves tested. For the calculations with the dialysand dilution factor V_1/V_0 the mean value of estimates for each series was taken. At first sight it would be the better approach if for each cell the individual estimates of V_1/V_0 were used instead. It appeared however that the variation in c_1 within one series was much smaller than the variation in V_1/V_0, so that the latter approach would introduce additional noise. On the other hand, using means of V_1/V_0, one runs the risk of introducing a systematic error. This may be the cause of the observation of some recoveries significantly differing from 100%, although the diffe-

rence does not mean much in an absolute sense. The effect of dialysand dilution was tested on cortisol in addition to aldosterone to exclude the possibility that the observed ineffectiveness of dialysand dilution on free hormone would be a mere consequence of the linear dilution effect. The finding of complete absence of any effect of dialysand dilution on free cortisol shows that also when the hormone-protein equilibrium does not provide a simple relationship between free hormone and dilution, the principle is valid, as was anticipated in ch.I.5.

The linear dilution effect on the f/b ratio for aldosterone and the non-linear effect in the case of cortisol with true dilution could be readily demonstrated. For cortisol a direct assay was employed, which is equally well suited for estimation of free hormone fractions as the indirect method, but eliminates all possible effects of tracer contamination. This was expected to play a negligible role in the free aldosterone assays.

The theoretically and experimentally proven equivalence of the estimates f_0' via eq.(IV-4) which employs c_2 and c_0 and eq.(IV-9), which may be considered as the Slaunwhite procedure corrected for dialysand dilution, makes it possible to test the validity of both methods at a time by examining the dependence of the former, less cumbersome approach, on true sample dilution.

It is by virtue of the linear dilution effect on free aldosterone, that estimates of the free fraction corresponding to a certain dilution while measuring at a different dilution using (IV-4), yield the same value as the true free fraction calculated by (IV-3) from measurement at the correct dilution factor. The results of free cortisol estimates demonstrate that in the general case it is not possible to obtain an accurate estimate of the free fraction corresponding to a certain dilution while performing the assay at a different (true) dilution. This means that also the estimate f_0' according to (IV-9) which is identical to the free hormone fraction within the dialysand, as it would be if no dialysand dilution had occurred, also in the case than undiluted serum has been dialysed, has no general validity. At first sight this may seem inconceivable since any difference between the original sample and the dialysand in which no dilution has occurred is not readily recognised. However,

one has to realise that a certain amount of hormone has left the dialysand, so that the (valid) estimate of the free hormone fraction in the dialysand corresponds to a total hormone concentration different from the level in the original sample before dialysis. The circle is closed by the proof that this effect of loss of hormone into the dialysate with regard to the free hormone concentration is adequately described as and equivalent to a dilution process.

IV.6. Summary

In ch.IV.2 ways are outlined for obtaining from equilibrium dialysis data as close as possible approximations of the free hormone concentration or fraction corresponding to the original undiluted state of the sample (F_0 and f_0). The calculation procedures for this purpose are derived from the theoretical considerations in ch.I.4 and I.5. How close the estimate by these calculations approach the true values, depends on how far the linear dilution effect (direct proportionality of the free/bound ratio with dilution) exists. In practice the effect of dilution therefore has to be assessed first, in order to obtain an impression of the accuracy of the result (IV.2.1). No alternative to the calculation of F_0' as an estimator of F_0 from results of application of direct free hormone assay in equilibrium dialysate (ch.IV.2.1) was found in the literature, but with regard to evaluation of data from the indirect method, a number of ways can be found in the literature. The most important are the approach by Oppenheimer, by which the true free hormone fraction corresponding to the actual dilution of the sample to the total volume of the dialysis system is obtained, and the estimate according to Slaunwhite by which the actual free hormone fraction in the dialysand at equilibrium is obtained. It is demonstrated that neither of these methods, since they are based on data obtained in a diluted state, can yield by any correction measure estimates of f_0 that have general validity. This is true also of the approaches proposed in this study, one of which has the merit of much easier assessibility since it does not require measurement of fluid shift, but is restricted of cases where tracer is quantitatively recovered.

The other is independent of recovery but requires assessment of fluid shift in addition to measurement of both dialysate and dialysand radioactivity. The finding of ch.I.5 that dialysand dilution caused by fluid shift due to osmotic pressure has no effect on the free hormone concentration as long as the ratio between the volume of sample assessed (V_0) and the total dialysis system volume (V_t) does not change, is verified experimentally in free aldosterone (IV.3.1) and free cortisol (IV.4.1) assays. Different dilutions of serum are assayed, while the true dilution is kept constant. For assessment of the effect of varying the true dilution on free aldosterone, an indirect method was used whereas free cortisol in different dilutions was directly assayed in the dialysate. The results confirmed a linear dilution effect for free aldosterone and no such simple relation for free cortisol. In accordance therewith, estimates of free hormone fraction employing correction for dilution based on the linear dilution effect gave accurate results for free aldosterone, but not for free cortisol.

CHAPTER V. FREE HORMONE ASSAY BY SYMMETRIC DIALYSIS: SOME BASIC EXPERIMENTS.

V.1. Introduction.

According to the theory developed in ch.II, symmetric dialysis would be an attractive alternative to equilibrium dialysis, although some deviations from ideal behaviour in terms of the rate-limiting step were anticipated. This chapter describes experiments that permit further evaluation of the possible advantage and drawbacks of the method and the mathematical models developed in the theoretical section.

The material presented forms by no means a complete experimental evaluation of all free hormone assays mentioned in this study, but it shows what elements such evaluation should contain and what problems may be expected. In this respect, very often reference will be made to the assay of free T4 by symmetric dialysis, which was studied more extensively.

The chapter first deals with the relation between tracer distribution and dialysis time, which takes the form of a "time curve', in order to ensure the validity of the dialysis rate as a measurement parameter (V.2). Thereafter, experiments concerning the relation between dialysis rate and free hormone fraction are studied (V.3). In V.4 the advantages of the method with regard to the independence of precision on free hormone fraction and low susceptibility towards tracer contamination, as anticipated in ch.II, are examined more thoroughly. Finally, some applications of free T4 and free T3 assays of clinical relevance are shown (V.5).

The appendix to this chapter deals with theoretical and experimental elaboration of the film diffusion concept and its role in symmetric dialysis, using experimental data from V.3.2.

V.2. Time dependence of tracer distribution.

V.2.1. Introduction.

According to the definition of the dialysis rate U of a symmetric dialysis process eq.(II-14); ln I = -U.t, where I is the ratio of the difference in tracer concentrations in first and second compartment at a certain point in time to total tracer present: $I = (c_1 - c_2)/(c_1 + c_2)$. It was also shown there that U = 2ADf/V, an expression which consists only of constants. Therefore plots of -ln I vs dialysis time should yield a straight line through the origin. This relation is tested first on a variety of ligand-binding protein combinations and cell constants. The position of the obtained curves, represented by the intercepts on the coordinates depends in the first place on the accuracy of the time scale, which in its turn depends on the way the process is started and ended. A variety of other processes influencing the position of the observed zero point can be thought of. E.g. there might be a mixing phase which may lead both to a delay (before tracer reaches the membrane) or to an initial apparent high speed of membrane penetration, if tracer is pipetted at the membrane surface and starts its transfer across before it has become evenly distributed throughout the half-cell. Therefore the influence of the route of administration of tracer to the dialysis system, which marks the start of the process, is also examined. A further possible effect, i.e. deterioration of the sample during dialysis, may also be of importance by affecting the linearity of the time curve, or the location of the intercept.

V.2.2. Tracer distribution vs. time curves.

Symmetric dialysis was carried out and ln I computed according to ch.III (paragraphs III.1.1 and III.1.3). Estimates of ln I were made on different points in time. Generally, for the shorter dialysis periods of a few hours or less, the different ln I estimates were obtained by adding tracer at different points in time and emptying

all cells at the same time. When longer dialysis periods were used, the end of the process was varied instead according to the length of dialysis periods required. Since addition of tracer to a cell takes much less time than emptying the cell this procedure was adopted for the short assays. With longer dialysis periods the start and terminating procedure is much less critical. For a number of samples complete "time curves" consisting of five or more points were made, for others tracer distribution measurements were made only at two points in time. For the complete curves the correlation coefficient, slope and intercept an the y (= -ln I) axis were calculated in addition to the standard deviation of ln I values about the regression line and the standard deviations of the estimated slope and intercept. The significance of the y-intercept difference from zero was tested also.

Table V.1 gives the more relevant data obtained by regression analysis of -ln I vs. time for different combinations of ligand, binding protein, buffer, membrane and cell dimensions.For the two-point estimates just slope and intercepts are given. Some of the data are graphically represented in figs.V.1 (large time-scale) and V.2 (short time-scale). Although no general criteria for linearity can be given, high correlation coefficients and concomitant. narrow confidence intervals for the slope of the regression line (=dialysis rate) suggest a linear relation between ln I and time. The fundamental equations describing the symmetric dialysis process require that the regression lines go through the origin. In some instances however the y-intercept differed significantly from zero. Artefacts, viz. tracer impurities or difficulty in defining accurately the point in time which marks the start of the process are considered to be likely explanations. Actually, for a number of cases from table V.1 tracer contamination was demonstrated which could account for a significant or even major proportion of the effect (ch.V.4.2). Accurate determination of the zero point (t = 0) which defines the location of the time-scale obviously depends also on the events in the very first phase of the process, particularly those of mixing of tracer with the half-cell contents. Therefore the effect of the route of tracer administration was tested on 1 : 50 diluted pool-serum with T4 as the labelled ligand in 1 ml cells, and with undi-

Ligand	Medium	r	s	slope ± SD (dialysis rate, h^{-1})	(y)-intercept ± SD
T4	phosphate b.1)	0.9881	0.09675	2.884 ± 0.1836	-0.09948 ± 0.08604
	" 2)	0.9859	0.04356	1.624 ± 0.09743	-0.01557 ± 0.07434
	" 3)	0.9903	0.5529	2.645 ± 0.1402	0.02635 ± 0.04308
	" 4)	0.9980	0.02665	6.247 ± 0.2289	0.04044 ± 0.03206
	" 5)	0.9988	0.00440	0.4863 ± 0.01392	0.01860* ± 0.00426
	poolserum 1:50	0.9954	0.03694	0.04211 ± 0.00143	0.05322* ± 0.01937
	" 60°C	0.9959	0.05865	0.1048 ± 0.003868	0.4704 ± 0.03565
	" barb.	0.9987	0.03280	0.1030 ± 0.002347	0.08990 ± 0.02306
	" 1:20			0.01432	0.08020
	" 1:10			0.009305	0.02771
	" 1:5			0.004463	0.02996
	" 1:2			0.002522	0.01315
	" 1			0.0009884	0.01634
	H.S.A. 0.1%			0.1690	0.03013
	" 0.05%			0.3645	-0.00830
	" 0.02%			0.7354	0.1065
	" 0.01%			1.4826	-0.0219
T3	phosphate b.	0.991	0.02120	2.688 ± 0.06703	-0.00317 ± 0.02223
	poolserum 1:50'	0.990	0.009014	0.3461 ± 0.007508	0.03712** ± 0.00757
	" 1:50'	0.9989	0.007402	0.3509 ± 0.009363	0.1467*** ± 0.008044
	H.S.A. 1%			0.1579	0.02311
	" 0.5%			0.3127	-0.001653

Table V 1

Ligand	Medium	r	s	slope ± SD (dialysis rate)	(y)-intercept ± SD
Aldo-sterone	phosphate b.1)	0.9792	0.1166	6.325 ± 0.7560	-0.1079 ± 0.1385
	" 2)	0.9995	0.01722	4.635 ± 0.08170	0.07814 ± 0.01806
	poolserum	0.9865	0.009469	1.878 ± 0.1800	0.08904 ± 0.1898
cortisol	phosphate b.	0.9990	0.0314	6.310 ± 0.1615	-0.01371 ± 0.03130
	poolserum 1:4			1.562	0.04066
	" 1			0.6295	0.02480
methyl-blue	phosphate b.	0.9980	0.02950	2.563 ± 0.02288	-0.1135 ± 0.03356

Table V 1

Tracer distribution as a function of time.

Shown are results of linear regression analysis of the relation between - ln I ($I = (c_1 - c_2)/(c_1 + c_2)$) and dialysis time for different combinations of radioactive ligands; membranes, cell dimensions, rotation speed and solutions with or without binding proteins. Where no correlations coefficient and standard deviation are given the ln I measurements were done at two points in time, whereas for the other assays as a rule measurements were done at five different points in time.

Levels of significance: *: $p < 0.05$, **: $p < 0.02$, ***: $p < 0.01$.

Table V.1. Explanation:

T4

Phospate buffer: 1) Spectrapor 2 membranes, 1 ml cells 8 rpm.
2) Diachema membranes, 1 ml cells, 8 rpm
3) Visking membranes, 1 ml cells, 20 rpm.
4) Spectrapor 2 membranes, 0,2 ml cells, 8 rpm.
5) Visking membranes, 1 ml cells, 20 rpm, phosphate + 0.04% Triton X100.

Poolserum 1:50 diluted in phosphate buffer: Visking membranes, 1 ml cells, 20 rpm.

60° C: the same, but heated for 2 hrs before dialysis.

Barb.: the same, but diluted in barbital buffer, 0.075 M, pH 8.6.

H.S.A. 0.1 - 0.01%: dilution in phosphate buffer, Visking membranes, 1 ml cells, 20 rpm.

T3

Phosphate buffer: Visking membranes, 1 ml,cells, 20 rpm.

Poolserum 1:50 diluted as with T4,but with tracer: ^{125}I-T3 Abbott,

Poolserum 1:50', the same but with tracer: ^{125}I-T3 NEN.

H.S.A. 1% and 0.5%: as with T4.

Aldosterone

Phospate buffer: 1) Visking membranes, 0.150 ml samples in 0.2 ml cells, 20 rpm.
2) Diachema membranes, 0.200 ml samples in 0.2 ml cells, 8 rpm.

Poolserum: as for buffer, see fig.V.2.

Cortisol

All three assays: Visking membranes, 0.150 ml samples in 0.2 ml cells, 20 rpm.

Poolserum diluted 1:4 in phosphate buffer, and undiluted.

Methyl Blue

Visking membranes 1 ml cells, 8 rpm.

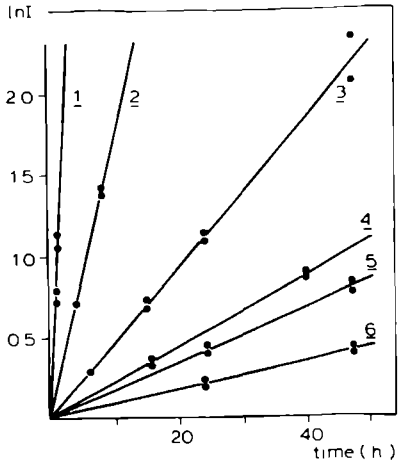


Fig.V.1.

Some symmetric dialysis "time curves" plot of -ln I vs time for the following combinations: 1) ^{3}H aldosterone in undiluted serum, Spectrapor 2 membranes, 1 ml cells, 8 rpm.
2) T4, 0.1% HSA, see table V.1.
3 - 6 T4, poolserum diluted 1:50, 1:25, 1:20, 1:10 in phosphate buffer, Spectrapor 2 membranes, 1 ml cells, 8 rpm.

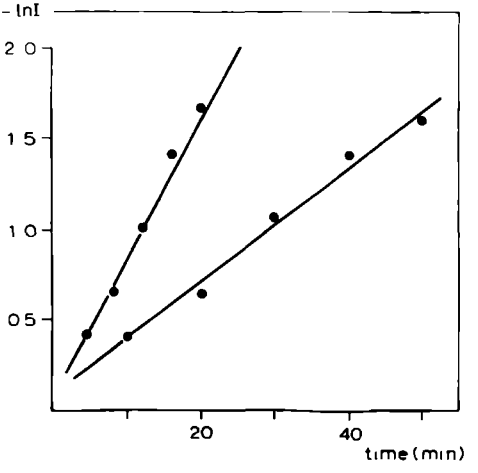


Fig.V.2.

Symmetric dialysis time curves of ^{3}H aldosterone in phosphate buffer (upper curve) and poolserum (lower curve).
Conditions: Diachema membranes, 0.200 ml samples in "micro" cells, 8 rpm.
For slopes and intercepts see table V.1.

luted serum with ^{3}H cortisol as the ligand in 0.2 ml cells. Values of ln I, obtained after 16 hrs for T4 and 1.5 hrs for cortisol, when tracer was added by means of a 10 µl repeating dispenser directly into the cells or by prior incubation of tracer with the sample were compared. When T4 tracer was added to 1 : 50 diluted serum prior to placing the sample in the dialysis cell, a dialysis rate (h^{-1}) of 0.03582 ± 0.000893 S.D. (2.5%), n = 5 was found while with tracer addition directly into the cell it was 0.03725 ± 0.001018 (2,7%) n = 4. The difference was not secured statistically although suggested ($0.05 < p < 0.1$). Such a difference will easily go undetected with simple duplicate determinations since it corresponds to a 1.3% difference in I and hence also in the corresponding number of counts. No significant effect was observed for the parallel test employing cortisol tracer. Undiluted serum and micro cells either: U = 0.3767 ± 0.05760 h^{-1} for direct addition in the cell and U = 0.3266 ± 0.03514 h^{-1} after preincubation of the sample with the tracer. Here the rather short dialysis time (1h) causes a higher variation in assay results since the starting and ending manipulations as a source of random error are relatively more important, so that it is doubtful whether such a difference can be statistically established at all.

Tracer is added by means of a syringe of which the needle ends very near the membrane surface. Therefore the observations are interpreted as the consequence of transfer of a small proportion of tracer through the membrane before complete mixing, which is a prerequisite for binding to proteins, has taken place. This would result in an apparent elevation of the dialysis rate, when measurements are made at only one point in time, and in an upward shift i.e. a positive y-intercept of the time-curve if estimates of ln I are made at different points in time. The magnitude of this effect is expected to depend on viscosity of the sample and rotation speed. This would be supported by the observation that high, positive y-intercepts combine frequently with high serum concentrations at the low rpm setting. However, no such evidence can be obtained from table V.1.

Negative y-intercepts could be the consequence of a certain delay involved in establishment of a (tracer) concentration gradient

within the membrane (Helfferich 1962).
In general, significant upward or downward shift of the time-curve will lead to erroneous estimates of the dialysis rate, when measurements are done at one point in time. The construction of a time-curve therefore is a necessary prerequisite before performing dialysis rate measurements in a certain system and particularly the possibility of tracer contamination should be considered.

V.2.3. The stability of serum samples during dialysis.

The stability with time of undiluted serum being dialysed was tested with regard to its T4 binding in the following way.
Samples of poolserum were thawed with 24 hrs intervals and dialysed in undiluted form. Four days after the first sample had been placed in the cell, the process was stopped and aliquots of the serum corresponding to 96, 72, 48 and 24 hours dialysis were taken and fifty times diluted. In the samples thus obtained the free T4 fraction or the dialysis rate, which was sufficient for this purpose, was determined and compared with the dialysis rate in freshly thawed and diluted serum. The whole experiment was done with and without addition of sodium azide (0.001 M) to the undiluted serum. The effect of dialysing undiluted poolserum during different periods of time on the dialysis rate of T4 is shown in table V.2. It must be concluded that dialysing at 37°C for up to 96 hrs has no effect on T4 binding. Surprisingly, in the presence of sodium azide, an agent deliberately added to inhibit bacterial growth and deterioration resulting therefrom, there seems to be a slight increase in free T4 fraction measured in a 1 : 50 dilution, particularly when also a measurement after 120 hrs, which was not performed on samples without azide, is included. Then a significant correlation of free T4 fraction and time is found ($r = 0.8537$).
These results indicate that it should be safe to dialyse undiluted serum for some days, a period that will be needed in such a case to obtain precise measurements of tracer distribution.

Time at 37°	0	24	48	72	96	120 hrs
U	0.03799 ± 0.00243 (SD) n=8	0.03928	0.03823	0.03630	0.03972	
U (azide added)	0.03697 ± 0.00293 (SD) n=4	0.03876	0.03659	0.03991	0.04064	0.04523

Table V.2.

Test for time stability at 37° C of T4 binding in serum.
Undiluted serum with or without added sodium azide was rotated in dialysis cells at 37° C for different lengths of time, after which the dialysis rate U as representative of the free T4 fraction was determined in 1:50 diluted aliquots of the treated sera.

V.3. The relation between dialysis rate and free hormone fraction; relevance of the film diffusion effect.

V.3.3. Introduction.

Ideally, when only the diffusion of tracer through the membrane governs the rate of tracer transfer in a symmetric dialysis process, the dialysis rate U as defined in ch.II.3 eq.(II-14) is equal to the product of the free hormone fraction and the cell permeability constant $2AD_m/hV$. Knowledge of this constant would permit a straightforward determination of unknown free hormone fractions from assessment of dialysis rates. The cell permeability constant, in its turn, would be readily assessible from dialysis rate measurement in a solution of which the free hormone fraction is known, e.g. f = 1 for a solution without binding protein. In ch.II.4 it was shown however, that the direct proportionality of U and f disappears when processes other than membrane diffusion influence the rate of tracer transport. A new expression for the dialysis rate was derived (eq. II-23) which takes into account the possibility that diffusion of tracer in the unstirred liquid films adhering to the membrane influences the rate of tracer transfer. From this expression follows for the cell permeability constant or factor $U/f = 2A/V(2\delta f/D_\ell + h/D_m)$, which is a function still of f, and therefore not a constant. It is readily seen that if the film diffusion term $2\delta f/D_\ell$ can be neglected compared with h/D_m, the cell permeability factor assumes its ideal value of $2AD_m/hV$ so that U and f are directly proportional. The magnitude of the film diffusion term can be influenced experimentally by varying f and by varying the efficiency of agitation, on which the film thickness δ depends. Agitation efficiency depends on the speed of the cell rotating device but also on the filling level of the cells. If the mathematical description eq.(II-23) is correct, the effect of varying agitation efficiency on the dialysis rate or on the cell permeability factor depends on f, in such a way that the effect is smaller and eventually disappears when f is lowered. The reverse would also be the case: at higher agitation efficiency, the direct proportionality of U and f is extended towards higher values of f. In this section, the interrelation of

agitation efficiency and free hormone fraction are studied experimentally. First the agitation efficiency is varied by means of the levels of the solutions in the cells. The effect of this variation on the dialysis rate of T4 tracer is assessed at high and low values of f. A more extensive study is carried out by plotting ln U vs. ln f at two different rotation speeds. For this experiment solutions of different concentrations of human serum albumin (HSA) with added T4, of which f values were known, were used. In the appendix to this chapter, these ln U vs. ln f curves are further exploited for substantiation of the film diffusion concept in symmetric dialysis.

Possible rate-control by dissociation and association reactions between hormone and binding proteins was studied with antisera directed against aldosterone. Antisera were used since it appeared from the literature (Hillier 1975, Dixon 1968, Westphal 1977) that normally occurring serum binding proteins generally react very fast with their hormonal ligands, fast enough for not influencing the relatively slow tracer transfer through the membrane. Also in the case of the antisera no such effect was found in preliminary experiments, which was interpreted according to ch.II.4.3 as a consequence of the low free fraction of the solution tested which keeps the membrane diffusion slow. These studies form a part of the doctoral thesis of A.J.M. de Man (in preparation) and will be discussed more extensively there.

V.3.2. The influence of agitation efficiency.

Both eqs.(II-14) and (II-23) predict a direct proportionality of dialysis rate U with membrane area A and an inverse proportionality with sample volume. This means that for constant f the product UV/A will be independent of volume and membrane area. This is supported by the observation of a dialysis rate of 2.884 h^{-1} for T4 tracer in phosphate buffer in "macro" cells with A = 4.5 cm^2 and V = 1.0 ml, and a dialysis rate of 6.247 h^{-1} in "micro' cells, with A = 2.0 cm^2 and V = 0.21 ml, so that UV/A values were 0.641 and 0.656 respectively. Table V.3 gives dialysis rates of T4 tracer

in buffer and in 1 : 50 diluted poolserum at different sample volumes (0.75 ml, 1.00 ml and 1.25 ml) in "macro" cells. The dialysis rates in diluted serum, when multiplied by their corresponding assay volumes, are equal, which is in accordance with the above considerations, as are the observations in buffer for the 0.75 ml and 1 ml volumes. The dialysis rate in 1.25 ml buffer multiplied by volume, however, does not yield the same value. Since it was shown that the effect of volume changes generally follows the predicted pattern, it must be concluded that another factor has come into play. From the total half-cell volume (1.36 ml) it is found that 1.25 ml occupies 91% of the avalable space, whereas it is only 74% for 1 ml solution. The results suggest that the high filling level makes the agitation of the solution less efficient, so that thicker unstirred liquid films adhere to the membrane than at lower filling levels. Thus, besides an increase of V, also δ is increased which results in a lower cell permeability factor. The absence of such an effect in the diluted serum sample is fully consistent with this view, since the low value of f (about 0.008) has made the film diffusion term $2\delta f/D_\ell$ so small that a change in δ has no effect on the cell permeability factor. Additional support for the validity of this interpretation in terms of the film diffusion concept was found in the observation (not shown here) that influencing agitation efficiency by means of changing the speed of the rotating apparatus from 20 to 8 r.p.m. had no effect on the dialysis rate in diluted serum but reduced the dialysis rate in buffer by some 30%.

When the above results are interpreted in terms of the film diffusion concept, the conclusion must be drawn that there is a range of values of f for which the film diffusion term is still negligible, so that the cell permeability factor is indeed a constant. This was examined by measuring the dialysis rates at 8 and 20 r.p.m. rotating speed in human serum albumin (HSA) solutions of different concentrations containing T4 tracer (table V.4) of which the corresponding f values were known from equilibrium dialysis followed by RIA measurements on four of these solutions. When eq.(II-23) is written in logaritmic form:

	Buffer		Poolserum	
V	U	U.V. (± SEM)	U	U.V. (duplicate means)
1.00	2.27	2.27 ± 0.068	0.0430	0.0430
0.75	3.00	2.25 ± 0.074	0.0613	0.0453
1.25	1.44	1.80 ± 0.105	0.0343	0.0433

Table V 3

The influence of cell filling level on the dialysis rate. "Macro" cells (total capacity 1,36 ml) are filled with different volumes of buffer or 1:50 diluted poolserum, and the dialysis U of ^{125}I T4 is measured and corrected for volume (Visking membranes, 20 rpm).

H.S.A. conc.(%)	f	ln f	U	ln U	U	ln U
			8 rpm		20 rpm	
0.5	0.006678	-5.009	0.03998	-3.219	0.03810	-3.269
0.2	0.01653	-4.103	0.0918	-2.406	0.1051	-2.253
0.1	0.03252	-3.426	0.1645	-1.805	0.1823	-1.702
0.05	0.06299	-2.765	0.3182	-1.145	0.3231	-1.130
0.02	0.1439	-1.939	0.6765	-0.3908	0.7826	-0.2451
0.01	0.2519	-1.380	1.382	0.3232	1.4551	0.3751
0.005	0.4020	-0.9113	1.716	0.5400	2.215	0.7951
0.002	0.6720	-0.4669	1.823	0.6005	2.262	0.8160
0	1	0	2.084	0.7340	2.335	0.8481

Table V 4

Measurements data of dialysis rates of ^{125}I T4 in different solutions of H.S.A. in phosphate buffer at two different rotation speeds (Visking membranes, "macro" 1 ml cells). Data are graphically represented in fig.V.3.

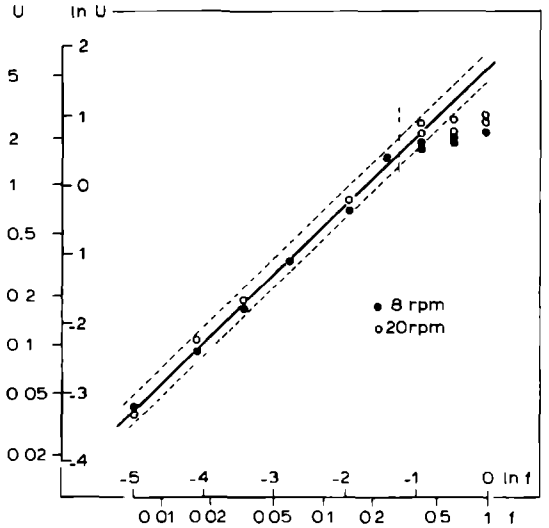


Fig.V.3.

Double logarithmic plot of dialysis rate U vs free hormone fraction f for T4 tracer in H.S.A. solutions at two different agitation speeds. Data points for the six lowermost values of f, on which linear regression was calculated, are duplicate means, whereas the other points are the individual ones. Broken lines represent the 95% confidence interval of a single estimate of U. Further data in table V.4.

$$\ln U = \ln f + \ln(2A/V) - \ln \left(\frac{2\delta f}{D_\ell} + \frac{h}{D_m}\right) \qquad (V\text{-}1)$$

It is readily seen that a plot of ln U vs. ln f will yield a straight line with slope 1 as long as (e.g. with low f) the film diffusion term is negligible. Such a plot is shown in fig.V.3 for both 8 and 20 r.p.m. For the six highest HSA concentrations (0.5 - 0.01%) at both r.p.m.'s a linear relation between the logarithm of dialysis rate and free fraction was found, with correlation coefficients of 0.9980 and 0.9978 respectively. The slopes of these first parts of the complete curve were 0.9599 $\pm$ 0.01915 and 0.9778 $\pm$ 0.02027, values which did not differ significantly from 1. Since the dialysis rates at these six concentrations did not differ for the two rotation speeds the results were pooled and a common ln U vs. ln f curve was obtained with r = 0.9974 and a slope of 0.9719 $\pm$ 0.01508 (not different from 1). A 95% prediction interval*) for estimations of U could be calculated (plotted in fig.V.3).
The data points of 20 r.p.m. corresponding to the next higher value of f (0.005% HSA) are shown to fall within these limits, whereas for 8 r.p.m. one of the duplicates falls outside. At still higher f, both for 8 and 20 r.p.m. the data points fall outside the prediction limits based on the low f values. Therefore it is concluded that at 8 r.p.m. film diffusion begins to play a significant role somewhere above f = 0.25 whereas for 20 r.p.m. it is still negligible at f = 0.40.
The linear part of the curve is represented by eq.(V-2), which follows directly from (V-1) by leaving out the film diffusion term:

*) It must be noted that the prediction interval gives the range in which 95% of the observations of ln U, made on a sample with a particular f, will lie. It must not be confused with the confidence interval for the curve which represents for each value of ln f the confidence interval of the expected value of ln U in terms of the relation between ln U and ln f obtained by linear regression analysis.

$$\ln U = \ln f + \ln(2A/V) - \ln(h/Dm) \qquad (V\text{-}2)$$

From eq.(V-2) it can be seen that the intercepts of the linear part of the curves with the ln U = 0 and ln f = 0 axes both give an estimate of $\ln(2AD_m/hV)$. The average value for $2AD_m/hV$ obtained thus is 5.12 h^{-1}. This value will be used in the further exploration of the film diffusion model (appendix VA).

V.3.3. Reference standards for symmetric dialysis.

As became apparent from the previous paragraph, direct proportionality between dialysis rate U and free hormone fraction f does not always hold. This means that it is not always possible to determine f for an unknown sample by simple division of measured U by a cell permeability factor, since this factor is not always a constant. The most rigorous approach to this problem is the construction of an U vs.f standard curve, analogous to the ln U vs. ln f curve in the previous paragraph. If measurements of unknowns are restricted to the lower, linear part of the standard curve, one reference standard solution with a known f in that lower range, which is assayed along with the unknowns, would suffice for calculating f from measured U for the unknowns.
The unknown f, f_x is then simply calculated by. $f_x = f_r U_x/U_r$. Measurements done in the non-linear region of the curve necessitate the use of more than one reference standard. In addition, these standards should have the same viscosity as the unknowns, since here measurements are made in a region where film diffusion, which depends on agitation efficiency which in its turn depends on viscosity, influences the dialysis rate. In both cases, construction of a complete standard curve is essential when setting up an assay, in order to be able to judge what standards are needed for the type of assay required. Suitable reference standards are stabile substances of which the free hormone fraction can be determined accurately by a method other than symmetric dialysis. Therefore the interaction between ligand and the binding substances should preferably be simple and well defined. For T4 as the ligand, HSA is

suitable for this purpose (table V.4, fig.V.3). With its (lowest) dissociation constant in the order of 10^{-6} mol/l, sufficient T4 can be added to determine free T4 in HSA dialysates by normal radioimmunoassay, at the same time not violating the $F < 0.01\ K_1$ condition. This condition is convenient since it allows the application of eq.(IV-4) for measurement of f_0 for each HSA concentration assayed and it also allows calculation of f for any other HSA concentration. Almost the same may be said for HSA with regard to T3, although a relatively high HSA concentration is needed to obtain sufficiently low free T3 fractions.

Standard curves for free cortisol and free aldosterone can not be made with help of pure HSA solutions, since this protein is a too weak binder for these hormones. One must resort to the use of serum instead. Although in the case of cortisol no linear dilution effect exists, it is possible to obtain true f_0 values for different serum dilutions in the same manner as described in ch.IV.4.2. By dialysis of an undiluted sample against an identical volume of buffer, application of eq.(IV-3) gives the f_0 value for twice diluted serum.

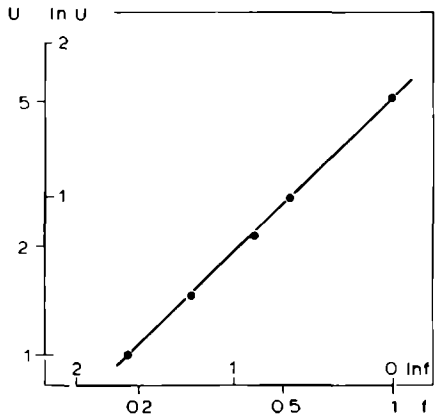


fig.V.4.

Double logarithmic plot of U vs f for ^3H cortisol in serially diluted normal poolserum. Assay conditions: Visking membranes 0.150 ml samples in "micro" 0.2 ml cells, 20 rpm.
Up to values of f = 1 (infinite dilution) a straight line is obtained (r = 0.9994, slope = 0.9845 $\pm$ 0.01953).

This is the same free fraction as the one prevailing in symmetric dialysis of twice diluted serum. It may be difficult to judge whether film diffusion still plays a role when determining the dialysis rate. In the T4-HSA system just described, the absence of any effect of changing rotation speed up to a certain value of f makes a film diffusion contribution very unlikely and it seems safe to take any HSA solution more concentrated than 0.01% as a primary reference when f has been determined by a reliable other method (equilibrium dialysis and RIA). In contrast, rotating speed had an influence on cortisol dialysis rate at any serum dilution up to 1 . 16 and pure buffer. Yet it was found (fig.V.4) that when 0.150 ml instead of 0.2 ml volumes are dialysed at 20 rpm rotating speed, a plot of ln U vs ln f yielded a straight line up to f = 1. Also in this case, values of f had been measured by equilibrium dialysis and RIA (cn.IV.4.2). This strongly suggests that the combination of high rotating speed, low filling level and a lower membrane diffusion coefficient prevents film diffusion control for all values of f. This experiment yielded a value for free cortisol in really undiluted serum, a condition that cannot be provided by equilibrium dialysis: 68.4 nmol/l for undiluted serum by symmetric dialysis, whereas the best estimate using equilibrium dialysis (i.e. F_0' based on dialysis of undiluted serum against an equal volume of buffer) gave a value of 60.1 nmol/l.
With aldosterone the situation is more complicated, since the free fraction of aldosterone in serum is so high that it does not sufficiently diminish the film diffusion term. Consequently viscosity begins to play a role, which manifests itself by a lower cell permeability factor for serum than for buffer.

V.4. Assay precision and accuracy.

V.4.1. Precision: Dependence of the coefficient of variation on ln I.

An essential feature of symmetric dialysis is the possibility of obtaining an estimate of the dialysis rate in principle at any point in time, by virtue of the direct proportionality of the logarithm of tracer distribution I with time. It is obvious that estimates of ln I made at the very start of the process, when only a small amount of tracer has arrived on the other side of the membrane or near equilibrium, when the tracer concentrations have almost become equal, will be less precise than measurements done when both a significant amount of tracer has passed the membrane and the difference between the radioactive concentrations is appreciable. In symmetric dialysis it is virtually always possible to reach that state by adaptation of the dialysis time to the dialysis rate. Thus the precision of the assay, which is most conveniently expressed by the coefficient of variation of a measurement, becomes independent of the free hormone fraction to be measured. In fig.V.5 it is illustrated how the dialysis time for a relatively high value of f is selected in order to obtain a value of I in a region of adequate precision. At lower values of f the dialysis time has to be prolonged to reach the same precision. The only restriction to such action would be a possible deterioration of the sample with a very long dialysis period. In fig.V.6 the coefficient of variation for the estimate of f is plotted as a function of I and $-\ln I$, using the following expression.

$$c.v.(f) = - (1/I \ln I)((1 - I^2)/c_0)^{\frac{1}{2}} \qquad (V\text{-}3)$$

which reflects the propagation of the counting error of c_1 and c_2 to the estimate of f. It was derived by means of general expression for error propagation (ch.III.2.3). For the case that 10.000 cpm of tracer are added, this figure shows that a c.v. of 3% or less is obtained for values of I between 0.2 and 0.75 which corresponds

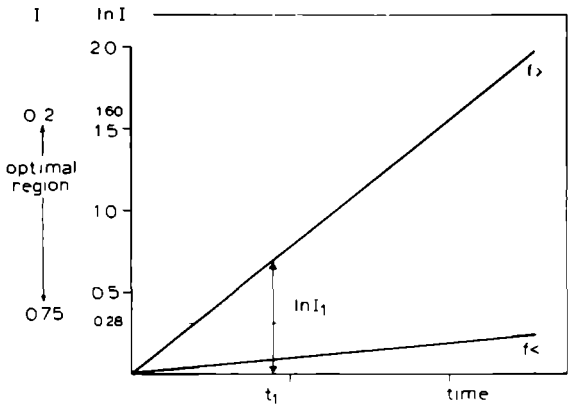


Fig.V.5.

Selection of dialysis time for optimal precision. Theoretical time curves for high and low free fractions are shown, and the range of values for I and -ln I for which the theoretical coefficient of variation is below 3%. For the sample with low f this region is reached beyond the time scale shown.

to -ln I values of 1.6 and 0.3. Highest attainable precision is at about I = 0.45 and -ln I = 0.8. These findings are always taken into account when setting up an assay.

With equilibrium dialysis it is not possible to adjust at will the amount of tracer transferred at the moment of the measurement. Precision depends on the tracer distribution at equilibrium and therefore it depends directly on f. With constant counting times, precision progressively falls with decreasing f.

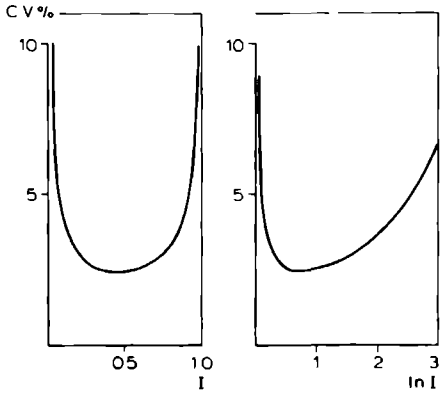


Fig.V.6.

Theoretical relation between I, -ln I and the coefficient of variation, according to eq.(V-3), when assuming that 10,000 cpm is added and 1 minute counting time.

Symmetric dialysis assay precision was assessed for 75 duplicate estimations of the free T4 fraction, obtained by overnight dialysis (mostly 17 - 18 hrs) of 1 : 50 diluted samples in 1 ml cells with added ^{125}I T4 tracer. These were divided into nine classes according to their ln I values, which ranged from 0.417 to 1.024. For each class the variation coefficient (c.v.) was calculated. For the mean ln I values per class also the theoretical c.v. was calculated according to (V-3) with c_0 = 15.000. The results are given in fig.V.7. The overall mean c.v. was 3.88 % (95% confidence interval: 3.28 - 4.52%) which is less than twice the overall theoretical value (2.05%). It must be concluded that within assay precision within this range of measurements is very good. Between assay variation coefficients (mean of duplicates) were 5.2% (n = 15) and 4.1% (n = 10).

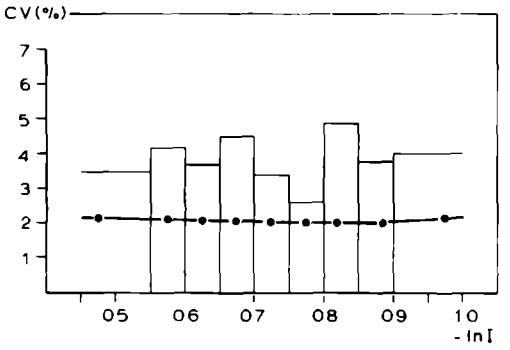


Fig.V.7.

Plot of coefficient of variation (c.v. %) against -ln I (vertical bars). Black dots represent the theoretical c.v. for the means of each class, calculated by means of eq.(V-3) with c_0 = 15,000 cpm. Data taken from 75 duplicate determinations of the free T4 fraction in 1:50 diluted serum samples (1 ml cells, Visking membranes, 20 rpm and dialysis for 18 hrs).

V.4.2. Accuracy: The effect of tracer contamination.

The accuracy of symmetric dialysis and equilibrium dialysis by the indirect method may be assumed to depend largely on the quality of the labelled hormone preparation. These preparations may include radiochemical impurities, which are expected usually to be less firmly bound or not bound at all in the sample. Thereby a distribution of radioactivity over free and protein-bound forms will result which is different from the distribution of the unlabelled and the (intact) labelled hormone. The effect of the presence of such interfering substances is dealt with most effectively by means of an example of the measurement of the free T4 fraction in a serum sample using a tracer preparation which contains besides T4 also some radioactive T3 and iodide.

Let an aliquot of a particular batch of T4 tracer to be added to a serum sample contain 10.000 cpm T4, 100 cpm T3 and 1000 cpm I^-. The sample had been diluted 1 : 50 and the free fractions of the components in that diluted sample are:

$$f_{(T4)} = 0.01$$

$$f_{(T3)} = 0.1$$

$$f_{(I^-)} = 1$$

First the effect on equilibrium dialysis is dealt with. If the sample is dialysed against an equal volume (1 ml) of buffer, at equilibrium the radioactivities in dialysand and dialysate have become, according to their respective free fractions:

	dialysand	dialysate	
T4	9901	99	cpm
T3	91	9	cpm
I^-	50	50	cpm

Now, in the terminology of ch.IV, considering the 1 : 50 dilution as the original sample, c_0 = 10,200 , c_1 = 10,042 and c_2 = 158 cpm/ml. Application of eq.(IV-4) gives:

$$f_0' = 0.0157$$

which is 57% too high.

When the buffer is replaced by the same 1 : 50 serum solution as is present as the dialysand, a symmetric dialysis system is obtained. Assuming a cell permeability constant D' of 1 and a dialysis time of 16 h the following result will be obtained:
When eq.(II-14) is written in terms of ln I by multiplication with t, and since D' = AD/V and $c_1 + c_2 = c_0$, one obtains

$$U.t = -\ln \frac{c_1 - c_2}{c_1 + c_2} = -\ln I = 2\, D'f\, t$$

from which ln I for the three components are found:

$$-\ln I_{(T4)} = 2 \times 0.01 \times 16 = 0.32$$

$$-\ln I_{(T3)} = 2 \times 0.1 \times 16 = 3.2$$

$$-\ln I_{(I^-)} = 2 \times 1 \times 16 = 32$$

the radioactive concentrations c_1 and c_2 of each component can be calculated using the above form of eq.(II-14):

	I	$c_1 + c_1$	$c_1 - c_2$
T4	0.7261	10.000	7261
T3	0.0408	100	4
I^-	1.3×10^{-14}	100	0
total	0.7123	10.200	7265

$(c_1 - c_2)/(c_1 + c_2)$ for total radioactivity now is 0.7123, from which the value of f which would be observed is calculated:

$$f = 0.0106$$

In this case the error is 6%.

Attention is drawn to the fact that under the above circumstances the less firmly bound T3 and the unbound I^- are almost evenly distributed over both compartments so that their contribution to the difference of radioactive concentrations is negligible. They only contribute to the total activity in the system and it is thereby that the end result is affected. The effect of such contaminants which only contribute to total activity can be calculated with help of a simple formula which is easily derived.
Suppose c_1 and c_2 are the amounts of radioactivity of intact tracer hormones in the first and second compartments, and that a radioactive amount ε of contaminant is present, which is bound so loose that by the time the dialysis process is stopped it is evenly distributed, so that each compartment contains 0.5ε of contaminant. Consequently, for the observed tracer distribution I' one obtains:

$$\ln I' = \ln \frac{(c_1 + 0.5\varepsilon) - (c_2 + 0.5\varepsilon)}{(c_1 + 0.5\varepsilon) + (c_2 + 0.5\varepsilon)} \qquad \text{(V-4)}$$

$$= \ln \frac{c_1 - c_2}{c_1 + c_2 + \varepsilon}$$

The tracer distribution I for the intact hormone is given by:

$$\ln I = \ln \frac{c_1 - c_2}{c_1 + c_2} \qquad \text{(V-5)}$$

Combination of (V-4) and (V-5) gives:

$$\ln I' = \ln I + \ln \frac{c_1 + c_2}{c_1 + c_2 + \varepsilon} \qquad \text{(V-6)}$$

The last term is for not too high levels of contamination equal to the fractional contamination ε'. ($\varepsilon' = \varepsilon/(c_1 + c_2)$). Eq.(V-6) also offers a simple way for correcting this type of contamination. When tracer distribution is measured both at time t_1 and t_2 (periods which must be sufficiently long to reach even distribution of contaminant), the difference of the observed ln I will be equal to the difference of the true ones, since ε' disappears by subtraction. So one finds:

$$\ln I'_{(t_1)} - \ln I'_{(t_2)} = - 2\ D'\ f\ t_1 + 2\ D'\ f\ t_2 \qquad \text{(V-7)}$$

from which the correct dialysis rate is calculated as follows:

$$\frac{\ln I'_{(t_1)} - \ln I'_{(t_2)}}{t_2 - t_1} = 2\ D'\ f = - U \qquad \text{(V-8)}$$

Of course with help of (V-8) and (V-6) it is also possible to measure ε'. For such an estimation as well as for the measurement of the true value for U the times t_1 and t_2 must not be too close (fig.V.8).
As the difference between t_1 and t_2 and the difference between ln I estimates at these points ar employed, this method for computing an accurate estimate of U will be referred to as "Δt-method".
Fig.V.9 shows time curves of two different ^{125}I-T3 preparations in 1 : 50 diluted poolserum. The slopes of the curves (dialysis rates) are 0.3461 $\pm$ 0.00750 and 0.3509 $\pm$ 0.00936 , which must be considered as identical, whereas the y-intercepts are 0.03712 $\pm$ 0.00757 and 0.14671 $\pm$ 0.00804 respectively. If these intercepts are considered to be the result of tracer contamination only, this contamination would amount, according to eq.(V-6), to 3.78% and 15.8% of T3 radio-

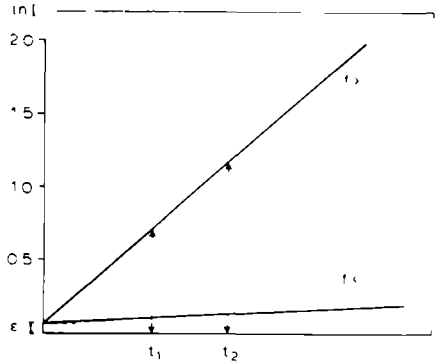


Fig.V.8.

Minimisation of tracer contamination effect.
Shown are theoretical time curves for high and low samples where the tracer is contaminated with a radioactive substance that is rapidly distributed evenly over both compartments, which results in a constant contribution to -ln I. The relative contribution to -ln I can be made smaller by increasing -ln I by prolonging the dialysis time. The contamination effect can be eliminated by division of the difference in ln I values obtained at two different points in time, t_1 and t_2, by $t_2 - t_1$ (Δt-method).

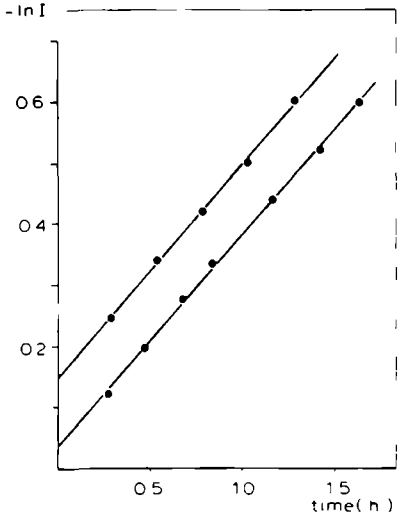


Fig.V.9.

Symmetric dialysis time curves for two different ^{125}I-T3 preparations in 1 : 50 diluted pool-serum. An elution pattern of the tracer used for construction of the upper curve is shown in fig.V.10. Further conditions: Visking membranes, 1 ml cells, 20 rpm.

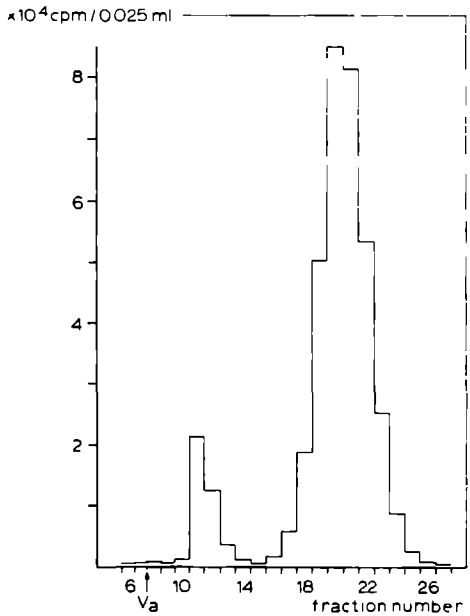


Fig.V.10.

Elution pattern of ^{125}I-T3 (Fig.V.9, upper curve) preparation. Conditions: Sephadex G25 Fine, column 25 cm x 1 cm^2, elution with 0.01 N NaOH at 0.5 ml/min., 2.5 ml fractions collected. Room temp.Va : Void volume.

activity. Column chromatography on Sephadex G 25 of the second tracer preparation demonstrated that radioactive iodide (11.3%) accounted for the larger part of the effect (fig.V.10). It may well be, since T3 tracer was directly added to the cells, that the y-intercept of the first preparation is due to a time-scale artefact. In that case the I^- contamination found would account for about 95% of the difference in y-intercepts found.

An impression of the problems arising from minute tracer contamination in equilibrium dialysis was obtained by overnight dialysing of 1 ml undiluted, 2x, 5x, 10x, 20x, and 50x diluted serum against 1 ml phosphate buffer. The percentages of total T4 radioactivity found in the dialysates (dialysable tracer Df'4%) and free f4 fraction f_0" after correction for dilution (simple division by dilution factor, see chs.I.4, eq.(I-18), IV.2.2, are shown in table V.5 and fig.V.11.
Apparently, the dialysate radioactivity with the undiluted serum consists only for a negligible proportion of intact T4 tracer, also at 50 times dilution the value found is still more than 100% higher than the "true" value, which was obtained by symmetric dialysis with 0.1% HSA as reference standard. The complete set of data from symmetric dialysis of the same solutions with the same tracer preparation is presented in table V.6 and fig.V.11.
The data in table V.6 and fig.V.11 show that the artefactual elevation of the assay results is very much limited as compared with the equilibrium dialysis results (table V.5). Furthermore it can be seen that with the longer dialysis period of 27.5 hrs the error is smaller, which is in accordance with theoretical findings (fig. V.8). Since the contribution of tracer impurity is relatively not too large, correction is possible. The proportion of contamination is readily estimated from the radioactivity in the equilibrium dialysate of undiluted serum since it was shown to consist almost completely of contaminating material (since 0.723% of total tracer was found in the dialysate and the sum of serum and buffer volume is twice the serum volume, the contamination amounts to 1.446%).
Fig.V.12 shows the free T4 fractions (f_0) with 18 and 27.5 hrs dialysis before and after correction for tracer impurity which,

Dilution factor	DfT4%	f_0" (%)
1	0·723	0.723
2	0.814	0.407
5	0.902	0.180
10	1.20	0.120
20	1.18	0.0592
50	1.80	0.0360

Table V 5

Results of measurement of free T4 fraction (DfT4%) by equilibrium dialysis without tracer purification of undiluted and diluted poolserum against an identical (1 ml) volume of buffer. (DfT4% via eq.(IV-3) and division by 2).

Dilution factor	time hrs	-ln I	fT4%	f_0" (%)
1	18	0.03413	0.0447	0.0447
	27.5	0.04352	0.0374	0.0374
2	18	0.05855	0.0767	0.0384
	27.5	0.08251	0.0708	0.0354
5	18	0.1103	0.1446	0.0289
	27.5	0.1527	0.1310	0.0262
10	18	0.1952	0.2559	0.0256
	27.5	0.2836	0.2434	0.0243
20	18	0.3227	0.4362	0.0218
50	18	0.6254	0.8200	0.0164

Table V 6

Results of measurement of free T4 fraction by symmetric dialysis using the same poolserum and the same ^{125}I-T4 tracer as in the experiment of table V.5. Another poolserum at 1 : 50 dilution (f_0 = 0.0160%) which had been calibrated against 0.1% H.S.A. was used as a standard.

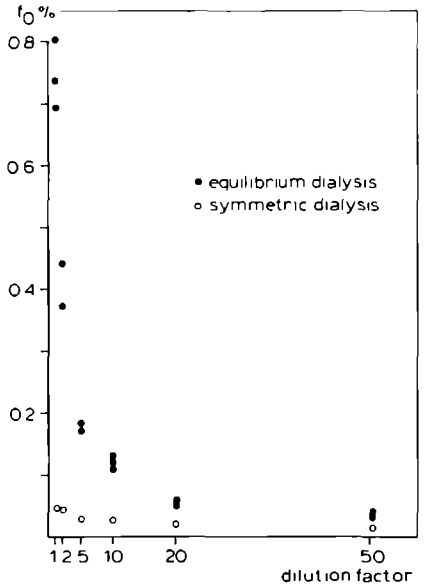


Fig.V.11.

Apparent free T4 fraction (f_0"%) of serial dilutions of poolserum, without tracer purification. Data from table V.5. (equilibrium dialysis) and table V.6. (symmetric dialysis). For fair comparison symmetric dialysis data from the 18 hrs. assay were taken since equilibrium dialysis was done for the same length of time. Both experiments conducted at 20 rpm in 1 ml cells with Visking membranes.

according to eq.(V-6) is done by subtracting 0.01436 from all values of ln I. Largely the same result is obtained when the Δt-method is used (table V.7).

Application of either of these methods is only possible when contaminant contribution to ln I is moderate. Inevitably it will be accompanied by loss of precision. Better results are expected when a simple and reliable purification step is applied in this stage. In the next experiment it was attempted to eliminate all tracer contamination effects in order to be able to measure free T4 in undiluted serum. Two measures were taken: first, dialysis time was extended to about 90 hrs, which appeared to be feasible by the results of stability tests in paragraph V.2.3 and second, intact T4 tracer on both sides of the membrane was captured by means of insolubilized antibody. Since unlabelled T4 exceeds the tracer amount and is identical on both sides of the membrane it is certain that identical amounts of T4 will be bound by the antibody, which of course cannot be added in an amount sufficient to bind all T4 present. Thus the amounts of radioactivity bound by the antibody permit calculation of the true value of ln I. Obviously this depends on the specificity of the antibody and the possibility to wash out all unbound radioactivity from the insolubilized antibody. This was tested by means of labelled T3 of which all activity was completely removed from the T4 antibody after three washings. Values for ln I obtained after 88.5 hrs for dilution factors 1, 2, 5 and 10 before and after the purification step are given in table V.8. From the data in table V.8 it appears that at these prolonged dialysis times tracer contamination forms a minor disturbance. At a ln I value of about 1 which is reached for the 1 : 10 diluted sample it has been reduced to a few percent, so purification for higher dilutions will be unnecessary. Finally the complete dilution series is given in fig.V.13. The corrected free fraction appears to decrease when diluting up to 10 times. At higher dilutions, the system behavesaccording to the model developed in ch.I and the Oppenheimer model.

An apparent decrease of the corrected free T4 fraction, which im-

Dilution factor	f_0"(%) 18 h	f_0"(%) 27.5 h	f_0"(%) Δt
1	0.0252	0.0244	0.0228
2	0.0282	0.0285	0.0290
5	0.0245	0.0232	0.0206
10	0.0231	0.0225	0.0214

Table V 7

Free T4 fractions corrected for tracer contamination after assesment of contamination (first two columns) or by Δt method. For further comment see text.

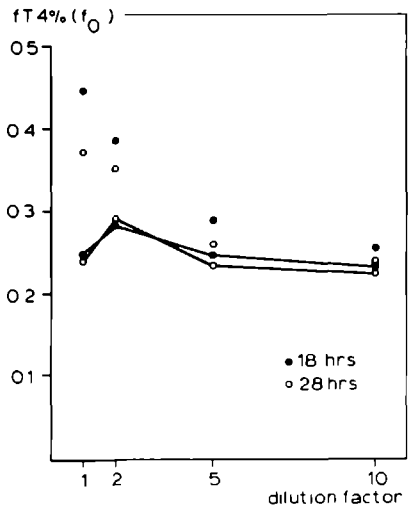


Fig.V.12.

The effect of tracer contamination of FT4% by symmetric dialysis. Connected points: values after correction for impurity.

dilution factor	-ln I ± SEM before	-ln I ± SEM after
1	0.1583 ± 0.00360	0.1363 ± 0.00652
2	0.2398 ± 0.01345	0.2268 ± 0.01288
5	0.4749 ± 0.01876	0.4462 ± 0.02322
10	0.9605 ± 0.03274	0.9554 ± 0.05310

Table V.8.

Log tracer distribution before and after tracer purification with solid phase antibody.

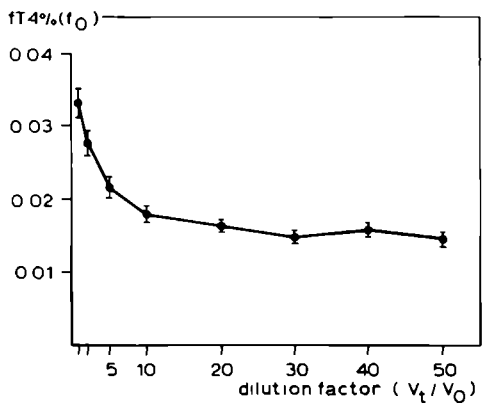


Fig.V.13.

Mean ± SD for fT4% (corrected for dilution) for serial dilutions of poolserum. Tracer purification after symmetric dialysis for varying periods by Immophase T4 insolubilised antibody.

plies the same for the free T4 concentration, for the lower dilution range, has been observed already with the earliest free T4 assays based on equilibrium dialysis with addition of tracer T4 (Ingbar et al. 1965, Sterling et al. 1966). Attempts were made to explain these observations in terms of tracer contamination (Schussler et al. 1967), or by the presence of a dialysable substance that binds T4 (Sterling et al. 1966). Later it was demonstrated that various anions (mainly H^+, Cl^- and HCO_3^-) are capable of increasing fT4% (Spaulding et al. 1972, Pedersen 1974, Sutherland 1975, Olsen 1979). The decrease in the chloride and bicarbonate concentrations when diluting with a medium not containing these anions, e.g. phosphate buffer would lead to a relatively increased binding and thus to a lower f14% than that expected from the dilution only.

In contrast to the well-documented effects of the anions, data about the actual disappearance of the so-called paradoxical dilution effect, when diluting while keeping these anions at a constant level, are relatively scarce. It was reported by Sterling (1966), although no data were shown, that the dilution effect disappeared when serum was diluted with its own ultrafiltrate. Pedersen (1974) showed that for a serum that had been predialysed against an excess volume of 25 mmol/l $NaHCO_3$ in 100 mmol/l NaCl, the paradoxical dilution effect, assessed by ultrafiltration did not exist when diluting with the same solution, whereas for the same sample the effect was strongly present when 0.067 mol/l phosphate buffer was used for dilution. Ekins (1975) showed for two different sera that diluting from a factor 12.5 onwards with a 0.01 M HEPES - 0.62% saline buffer did not affect the FT4 and the same was demonstrated by Weeke (1979) for dilution from a factor 28 onwards (dialysand dilution 1 : 4). These observations are of limited significance as to the paradoxical dilution effect because of its occurrence mainly with lower dilutions (Ingbar et al. 1965, Spaulding et al. 1972, Uchimura et al. 1976). The paradoxical dilution effect itself, although frequently described, has not been very accurately defined, because many authors refer to dialysand dilution instead of V_t/V_0 and in a number of cases the free T4 fraction was calculated as the ratio of dialysate and dialysand radioactivities while no correction for fluid shift was

carried out, so that the results may have become exaggerated (Spaulding et al. 1972, Uchimura et al. 1976). A straightforward comparison of fT4% values obtained in sera diluted with phosphate buffer or a physiological buffer can be found in a report by Irvine (1974). This author, using a gel equilibration technique, measured fT4% in 15 different sera diluted 1 : 100 with various buffers. The results showed that the values obtained when diluting with Krebs-Ringer bicarbonate (KRB) buffer were on the average only 12% higher than those obtained in isotonic phosphate buffer of 0.15 M. When this observation is combined with the present result, the absence of a paradoxical dilution effect in KRB buffer would be doubtful. On the other hand the effect observed is of the same order of magnitude as described formerly by Ingbar and others. It is evident that further study, in which the first step will be the use of a more physiological buffer, will be needed to shed more light on the compatibility of fT4 values obtained in diluted and undiluted serum. Symmetric dialysis may prove itself to be particularly suited for this purpose.

V.5. Some applications of free T4 and free T3 assays by symmetric dialysis.

In order to demonstrate the usefulness of the free thyroid hormone assays by symmetric dialysis, their application will be shown in two situations of deviating serum binding of the hormones: pregnancy and TBG-deficiency. For the free T4 assay also data obtained during thyroid disease are available.

V.5.1. Free T4 and free T3 in euthyroidism.

The routine assays of the fractions of free T4 and T3 were already described in ch.III as were the radioimmunoassays for total T4 and T3. The percentage free T4 (fT4%) in 33 healthy subjects, belonging to laboratory staff, ranged from 0.0110 - 0.0200% (mean $\pm$ S.D.: 0.0153 $\pm$ 0.00204). Total T4 ranged from 64 - 154 nmol/l (mean $\pm$ S.D.:

106 ± 24.0 nmol/l). For the concentration of free T4 (FT4) a range of 10.3 - 24.6 pmol/l was obtained (mean ± S.D.: 16.0 ± 3.81 pmol/l).

The percentage free T3 (fT3%) in the same group of euthyroid volunteers ranged from 0.150 - 0.257% (mean ± S.D.: 0.200 ± 0.0266). Total T3 ranged from 1.52 - 2.70 nmol/l (mean ± S.D.: 2.00 ± 0.333 nmol/l) and for the free T3 concentration (FT3) a range of 2.85 - 5.22 pmol/l (mean ± S.D.: 3.98 ± 0.643 pmol/l) was obtained.

There is no general agreement on the normal ranges for free T4 and free T3. A review of free thyroid hormone assays and corresponding normal values was recently presented by Ekins (1979). Normal ranges estimated by the indirect equilibrium dialysis method, of which the technique of Sterling and Brenner (1966) is the most widely used, tend to be considerably higher than the above values. Previous own experiments with a modification of Sterling's method (Hermann et al. 1971) in a group of 16 euthyroids with total T4 of 76 - 127 nmol/l yielded fT4% values of 0.014 - 0.030% with absolute free T4 levels ranging between 15.6 and 27.8 pmol/l. The same technique also may give varying results in different hands. This might reflect effects of tracer contaminations of diverse nature that are not removed by the magnesium chloride precipitation step. Such effects are absent in the method of Ekins and Ellis (1975) which was also employed by Yeo, Lewis and Evered (1977), in which the free hormone is directly radioimmunoassayed in an equilibrium dialysate. The same principle was also applied by Kristensen and Weeke (1977). However, also with this approach the results vary between laboratories. Ekins et al. report a normal FT4 range of 9.7 - 21.9 pmol/l, whereas Yeo et al. arrive at 4.05 - 16.65 pmol/l. Also for FT3 different normal ranges were found (Ekins et al.: 5.4 - 10.0 pmol/l and Yeo et al.: 4.5 - 15.7 pmol/l). These assays were performed at a 1 : 25 true dilution of the serum, and therefore they may be compared with the present results using 1 : 50 dilution since constancy of the free hormone concentration at these dilutions was theoretically and experimentally demonstrated in several instances (Oppenheimer et al. 1964, Spaulding et al. 1972, Uchimura et al. 1976 and own results not shown here). A further difference in assay conditions that may affect the results is the

buffer used to dialyse against and to dilute with, as already has been discussed (V.4.2).

V.5.1.1. Free T4 and free T3 in early pregnancy.

The rise in TBG and the concomitant rise of total T4 and T3 in pregnancy is well-known. Avrushkin et al. (1976) assayed total and free T3 and T4 concentrations and TBG capacity throughout pregnancy. Mean serum total T4 concentrations and TBG capacity rose in each successive trimester. Mean serum total T3 concentration was significantly elevated compared to that of normal subjects and did not increase further in either of the two ensuing trimesters. Serum free T4 and T3 concentrations were constant through pregnancy, free T4 being at the lower end of the normal range while the free T3 was significantly lower than normal. In contrast, Osathanondh et al. (1976) reported no difference in either total T4 or T3 levels between mid-pregnancy (15 - 20 weeks) and at term and no difference for free T4 and T3 levels for pregnant subjects and normals. In yet another report by Evered (1977) in which the free hormones were assayed directly, significant lower levels for both FT4 and FT3 were found during pregnancy.
Attention was drawn to the events in early pregnancy by the study of Exalto (1979). In 24 normal pregnancies total T4 and T3 and an index of free T4 were assayed from 6 - 16 weeks (menstrual age). A rise in total T4 and T3 was observed from 0 - 14 weeks, whereas the free T4 index remained virtually constant. In the present study, free T4 and T3 fractions were determined by symmetric dialysis in a subgroup of five of these subjects, and FT4 and FT3 were calculated. Figs.V.14a - f show total T4, total T3, fT4%, fT3%, FT4 and FT3 plotted against duration of pregnancy at two-week intervals. Fig.V.14a shows that up to 10 weeks, all but one total T4 values are still in the normal range. However, a significant rise of total T4 could be demonstrated already in the period of 6 - 10 weeks. Significant rises* were also found over the 4-week periods 8 - 12

* The suggestion of dr.van Elteren, Mathematisch-statistische advies afdeling, Fac.der Wiskunde en Natuurwetenschappen, to use the sum of Spearman rank correlation coefficients as a test statistic, is gratefully acknowledged.

and 10 - 14 but not in the period of 12 - 16 weeks. This suggests that a rise occurs in all but the last period 14 - 16, which is in accordance with Exalto's findings with the larger group of 24 subjects, so that in this respect the 5 subjects chosen are representative for the larger group. From fig.V.14b can be seen that for T3 at 6 weeks pregnancy all values lie above the mean of the normal range. After 10 weeks only one value is still in the normal range. Significant rises were observed in the periods 6 - 10 and 8 - 12 but not in the periods 10 - 14 and 12 - 16 weeks. The time course of the free T4 fraction is shown in fig.V.14c. The values at 6 weeks already tend towards the lower half of the normal range. At week 12 no value lies within the normal range anymore. The decrease in fT4% was significant in the intervals 6 - 10, 8 - 12 and 10 - 14 but not in 12 - 16. The fT3% (fig.V.14d) already drops below normal at 8 weeks of pregnancy. The decreases were significant in the 6 - 10, 8 - 12 and 10 - 14 weeks intervals, but not in the 12 - 16 weeks interval. The concentrations of T4 and free T3 (figs.V.14e and f) remain, with two exceptions for free T3, within the normal range. There is a slight tendency of free T4 values to fall in the lower part of the normal range. Significant increases or decreases were not found over the intervals tested. The limited number of data does not allow further inference to be made.
The above results suggest that the decrease in free T4 and T3 fractions is synchronous with the increase in total hormone levels. No evidence is obtained of absolute free hormone levels differing from normal or of any change of these levels during the first 16 weeks of pregnancy.

It may be concluded that the symmetric dialysis method is fully suited to follow the changes in thyroid hormone binding due to the TBG increase of pregnancy.

V.5.1.2. Familial TBG deficiency.

TBG-deficiency in man was first described by Tanaka and Starr (1959) who noted a complete absence of TBG binding of labelled T4 in the

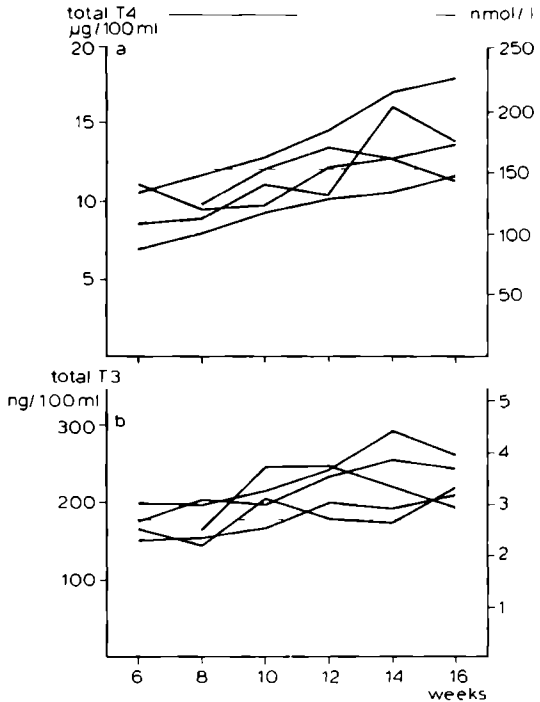


fig.V.14a, b

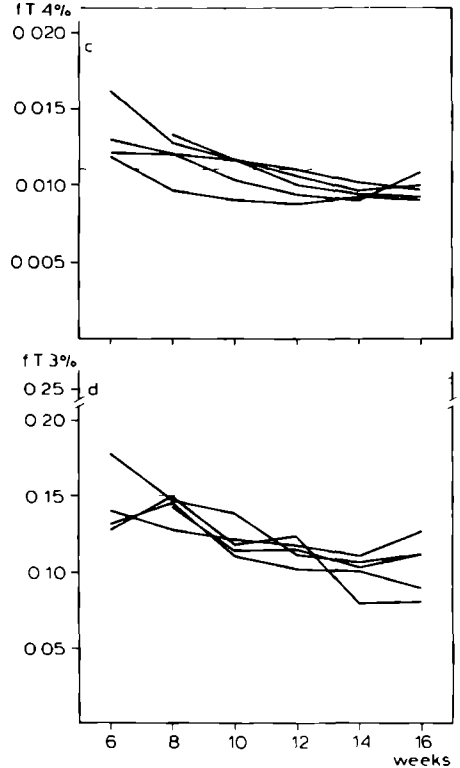


fig.V.14c, d

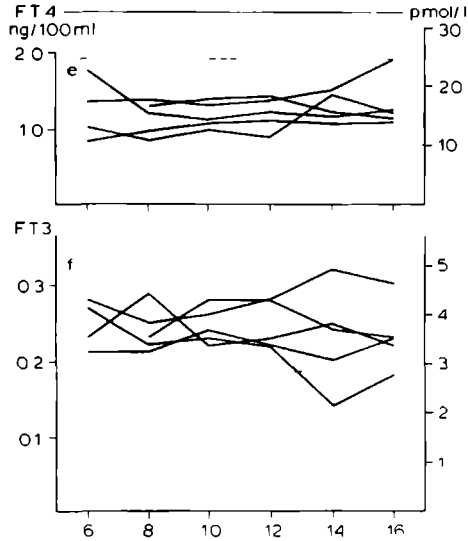


fig.V.14e, f

Fig.V.14a-f.

Total T4 (14a), T3 (14b), free T4 fraction fT4% (14c) free T3 fraction fT3% (14d), free T4 concentration fT4 (14e) and free T3 concentration FT3 (14f) in five normal subjects during the first 16 weeks of pregnancy.
Dotted lines represent upper and lower limits of the normal range.

electrophoretogram. Since then, both isolated cases and families with the trait have been described, in which TBG was either absent or low. Subjects with TBG deficiency are generally euthyroid (Konno 1975), and occasional observations of hypo- and hyperthyroidism must be considered as coincidential. Total T4 is lowered and concomitantly the free T4 fraction is increased. A number of investigators report normal values for FT4, whereas others find a decrease of free T4 concentration. More literature references concerning these observations can be found in a report by Premachandra (1976) on a subject with low TBG binding capacity but elevated FT4. Thus no unequivocal evidence exists as to FT4 in TBG deficiency, while data on FT3 were not found in the literature.

Here the results of a clinical study by Smals et al. (1979) obtained in two families of which 8 individuals (4 hemizygous and 4 heterozygous) had low or absent TBG, are summarised. Results of measurements of total T4, total T3, fT4%, fT3%, FT4, FT3 and TBG are given in table V.9. The latter was measured with a binding assay employing insolubilised antibody against TBG and T4 tracer (Immophase TBG, Corning), which was calibrated by comparison with TBG binding capacity assays by means of Scatchard plot analysis (normal range 212 - 357 nmol/l, n = 25).

For the complete group of 8 TBG deficient subjects, both total T4 and T3 were significantly lower* than normal ($p < 0.001$), while the free T4 and free T3 fractions were almost doubled ($p < 0.0001$) compared with normals. The mean free T4 concentration was found to be significantly lower than normal ($p < 0.01$), whereas FT3 was significantly elevated ($p < 0.001$). The deviations from normal tend to be more marked in the hemizygous individuals, although significance was only found in the case of FT4. The elevation of FT3 and the lowering of FT4 can also be represented by the FT3/FT4 ratio, which for the hemizygous group (0.47 ± 0.5) differed significantly from normal (0.23 ± 0.07). For all 8 subjects the ratio was 0.39 ± 0.11.

* Mann-Whitney test for unpaired observations

Subject	T4 nmol/l	T3 nmol/l	fT4% %	fT3% %	FT4 pmol/l	FT3 pmol/l	TBG nmol/l
hemizygous:							
G_1	31	0.74	0.028	0.60	8.7	4.4	0
G_2	36	1.00	0.032	0.58	11.5	5.8	18
G_3	28	0.89	0.037	0.62	10.4	5.5	0
F_1	31	1.61	0.029	0.32	9.0	5.2	-
mean	31.5	1.06	0.0315	0.53	9.9	5.2	
± S.D.	± 3.2	± 0.38	± 0.004	± 0.14	± 1.3	± 0.6	
heterozygous:							
G_4	64	1.47	0.026	0.34	16.6	5.0	120
G_5	33	1.29	0.036	0.37	11.9	4.8	-
G_6	54	2.10	0.022	0.26	11.9	5.5	-
F_2	48	1.09	0.026	0.31	12.5	3.5	77
mean	50.2	1.49	0.0275	0.32	13.2	4.7	
± S.D.	± 12.9	± 0.41	± 0.006	± 0.05	± 2.3	± 0.8	
overall mean	40.6	1.27	0.0295	0.425	11.6	5.0	
± S.D.	± 13.1	± 0.44	± 0.0052	± 0.148	± 2.47	± 0.74	
normal mean	106	2.00	0.0150	0.200	16.0	3.98	250
± S.D.	± 24	± 0.333	± 0.00204	± 0.0266	± 3.81	± 0.643	± 37.5

Table V.9.

Total and free thyroid hormone levels in TBG deficient members of families G. and F. Most values (except for TBG) are means of samples drawn at different times.

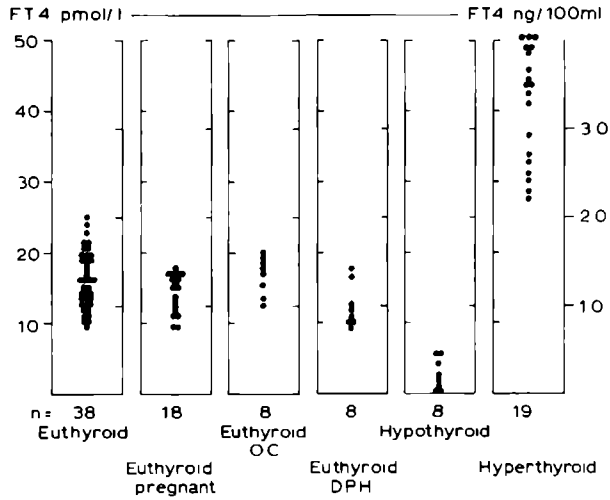
Since all 8 subjects were perfectly euthyroid, it appears that thyroid homeostasis is adequately maintained with lowered FT4 and elevated FT3 levels. This challenges the probably too simple view that the free thyroid hormone levels in blood are the sole determinants of thyroid status.

V.5.2. Free T4 and thyroid status.

Since a thorough clinical evaluation of the free T4 assay is beyond the scope of this thesis, assay results are shown for a number of defined, well-documented clinical subgroups only. The patient material was made available by drs.P.J.Derkinderen and J.H.H. Thijssen, Department of Endocrinology, University Hospital Utrecht for the sake of an interlaboratory comparison of free T4 assays of which preliminary results have been reported recently (Thijssen et al. 1979). In this study results of two commercially available methods, one consisting of separation of free hormone by means of gel filtration on Sephadex LH-20 and subsequent radioimmunoassay of the free hormone (Romelli et al. 1979) and the other relying on the uptake kinetics of T4 tracer by insolubilised antibody (Odstrchel et al. 1978), and the symmetric dialysis method, carried out in our own laboratory were compared.
FT4 measurements by symmetric dialysis were done on the following clinically well-defined groups:
Euthyroids (n = 38)
Euthyroid pregnants, > 20 weeks (n = 18)
Euthyroid on oral contraceptives (O.C.) (n = 8)
Euthyroids treated with diphenylhydantoin (DPH) (n = 8)
Primary hypothyroids (n = 8)
Hyperthyroids (n = 19)

The results are summarised in fig.V.15.

The following comments are made:
No overlap of FT4 values between euthyroid and pathological states is observed. Especially the discrimination with hypothyroids is



FT4 (pmol/l)						
mean	16.5	14.3	16.8	12.3		
± S.D.	± 3.91	± 2.83	± 2.67	± 3.06		
range	10.2-24.7	9.8-17.8	12.8-19.7	9.5-17.4	0-5.5	27.6-83

Total T4 (nmol/l)						
mean	106.8	159.9	144.7	83.0		
± S.D.	± 23.6	± 37.2	± 38.3	± 17.9		
range	64-154	95-215	78-203	67-112	0-46	137-338

Fig.V.15.

Free and total T4 in different clinical states.

abbrev.:

O.C.: subjects on oral contraceptives.

D.P.H.: Diphantoine treatment.

very good. Within the euthyroid range there seem to be downward shifts both for pregnant subjects and subjects receiving diphenylhydantoin, an agent known for its capability to displace T4 from its binding sites on TBG. In the latter case the mean differed significantly ($0.01 < p < 0.02$, Student's t test) from the mean of normals. The lower mean in the 18 pregnant subjects was not statistically secured ($0.05 < p < 0.1$), but the possibility that for larger groups the difference will be significant must be taken into account. However, FT4 of all three groups of euthyroid subjects which differ from normal as to T4 binding remain within the normal range. This contrasts with the observations on total T4, where only the 8 DPH treated subjects remain within the normal range, although the mean differs significantly from the normal mean. The results of the comparison with the other two free T4 assays and a free T4 index method will be published elsewhere.

V.6. Summary

In ch.V first some crucial points from the theory of symmetric dialysis developed in ch.II are closer examined by means of directed experiments. In ch.V.2 the time course of the natural logarithm of the tracer distribution is examined on a variety of combinations of radioactive ligands and binding protein solutions. In conformity with the theory, a linear relationship with time of which the slope represents the dialysis rate was found in all cases studied. Subsequently in V.3 the relation between the dialysis rate and the free hormone fraction was examined in view of the in ch.II introduced film diffusion concept; meant to explain the influence of measures, which have an effect on agitation of the cell's contents, on the dialysis rate. Agitation efficiency was decreased in two ways: by filling the cells to a higher level and by lowering rotation speed. In both cases these interventions lowered the dialysis rate in solutions with high free fraction, but had no effect for low values of f. Also in conformity with the theory, dialysis rate and free hormone fraction were found to be directly proportional for a long range of free fractions, where change of rotation speed has no

effect, but the curve deflects at higher free fractions and there rotation speed affects the dialysis rate. From these observations it is concluded that preliminary experiments before setting up an assay should include the construction of a dialysis rate vs. free hormone fraction standard curve in order to determine the linear range of the relation. By preference, assays should be carried out in this linear range and the results should be evaluated using a reference solution with a known free fraction within the linear range. The choice of the proper dialysis period for assessment of the dialysis rate depends on the precision with which the tracer distribution is determined (V.4). Thus it is possible to obtain the same precision virtually independent of the free hormone fraction, such in contrast with equilibrium dialysis. The within assay and between assay precision of the free T4 assay was assessed and found to be better than 5%. The effects of weakly bound and unbound radioactive contaminants (T3 and iodide) in a free T4 assay both by equilibrium dialysis and symmetric dialysis were theoretically analysed. This disclosed a much larger effect on the equilibrium dialysis results, which also could be demonstrated by experiment. A simple method could be derived to correct for the effect in symmetric dialysis, which involves measurement of tracer distribution at two points in time instead of one. This method was experimentally verified by means of free T4 and free T3 assays. Subsequently it was shown that the effect of tracer contamination can be minimised by prolonging dialysis time so that tracer purification becomes almost unnecessary. Finally it was shown that free T4 and free T3 assays by symmetric dialysis are useful for clinical studies. The changes in thyroid hormone binding during the first 16 weeks of pregnancy could be followed quite accurately. In TBG deficiency an elevation of free T3 and a decreased free T4 was observed, and the assays of free T4 concentrations and fractions discriminated well between various clinical categories.
In an appendix the validity of the film diffusion concept to explain almost fully all observed deviations from the simple model of symmetric dialysis is further explored.

Appendix VA

Further Exploration of the Film Diffusion Model.

The data on dialysis rates at different f and rotation speeds (table V.4) permit calculation of specific film diffusion parameters if first appropriate relations are derived from eq.(II-23). The first step is to write (II-23) in reciprocal form:

$$1/U = (1/f).(V/2A).\left(\frac{2\delta f}{D_\ell} + \frac{h}{D_m}\right) \qquad \text{(VA-1)}$$

Let the film thickness at 8 and 20 rpm be δ_1 and δ_2 respectively. Then the difference between the reciprocal dialysis rates at 8 and 20 rpm will be:

$$\Delta 1/U = (V/2A)\ \frac{2(\delta_1 - \delta_2)}{D_\ell} \qquad \text{(VA-2)}$$

According to ch.II.4.2, D_ℓ will be a function of f, since the diffusion coefficients in solution of free and bound hormone will differ appreciably. Substitution of (II-24) in (VA-2) and taking its reciprocal again gives:

$$(\Delta 1/U)^{-1} = (2A/V)\ \frac{fD_h + (1 - f)D_p}{2(\delta_1 - \delta_2)} \qquad \text{(VA-3)}$$

where D_h and D_p denote the diffusion coefficients in free solution of free and bound hormone respectively. Eq.(VA-3) can be rearranged to:

$$(\Delta 1/U)^{-1} = (A/V)\ \frac{D_h - D_p}{\delta_1 - \delta_2}\ f + (A/V)\ \frac{D_p}{\delta_1 - \delta_2} \qquad \text{(VA-4)}$$

This function attains for f = 0 the value $(A/V)(D_p/(\delta_1 - \delta_2))$ and for f = 1 it is $(A/V)(D_h/(\delta_1 - \delta_2))$ which makes it possible to obtain the ratio of D_h and D_p and, if one of the diffusion coefficients is known, the difference in film thickness $\delta_1 - \delta_2$. Furthermore, from the individual dialysis rates in pure buffer, where $D_\ell = D_h$, the individual values of δ_1 and δ_2 can be calculated from (II-23) or (VA-1), since $2AD_m/hV$ has been measured already (5.12 h^{-1}). From the dialysis rates at 8 and 20 rpm of T4 in the more diluted HSA solutions (0.05 - 0%) obtained from a separate experiment the corresponding values of $(\Delta 1/U)^{-1}$ were plotted against f (fig.VA.1). A correlation coefficient of 0.9705 was obtained and a slope of 8.1547 $\pm$ 1.019. The function values at f = 0 and f = 1 were 2.05 $\pm$ 0.53 and 10.21 $\pm$ 0.68 respectively, so that the ratio D_h/D_p became 4.97 $\pm$ 1.622.

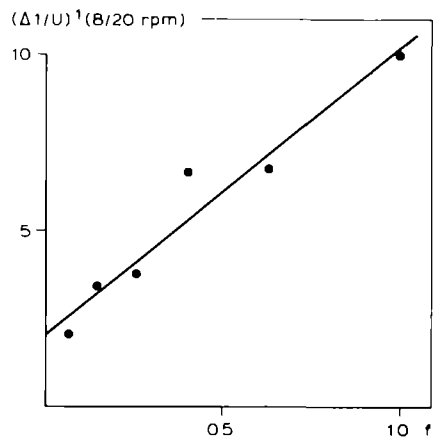


Fig.VA.1

Plot according to eq.(VA-4) in order to obtain the ratio of diffusion coefficients of free to bound hormone (D_h/D_p) from the intercepts at f = 1 and f = 0. The slope of the curve subsequently yields the difference of film thickness at 8 and 20 rpm agitation rate.

Since the diffusion coefficient is roughly proportional to the reciprocal of the cubic root of peptide molecular weight, this D_h/D_p ratio, taking 69.000 as M.W. for albumine, would predict a peptide molecular weight for the ligand and about 560. It does not seem unreasonable that the diffusion coefficient of T4 (which is not available in the literature) would correspond to that of a peptide with molecular weight in this range. If for the diffusion coefficient of albumin at 37°C value of 3.58×10^{-3} cm^2/h from the literature is adopted, the T4 diffusion coefficient would be 1.78×10^{-2} cm^2/h and $\delta_1 - \delta_2 = 7.58 \times 10^{-3}$ cm, as can be found by multiplication of the reciprocal of the slope (= 1/8.15) by $D_h - D_p$ (= 1.42×10^{-2} cm^2/h) and A/V (= 4.5 cm^{-1}).

This difference was also calculated from the individual film thickness δ_1 and δ_2 which are determined first by means of eq.(VA-1). This requires knowledge of the cell permeability constant $2AD_m/hV$ which was measured already (5.12 h^{-1}) and estimates of U at 8 and 20 rpm (2.18 and 2.79 h^{-1}) under conditions that the film diffusion contribution is maximal (f = 1). For δ_1 one finds 2.11×10^{-2} cm and for δ_2: 1.32×10^{-2} cm, so that their difference is 8×10^{-3} cm. The similarity of this result with that obtained above suggests consistency within the applied model. Apart from that, the film thicknesses found seem to be reasonable in view of literature values (Helfferich 1962).

By consequence of the wide confidence intervals, the above experiments do not permit precise calculation of film diffusion contribution to overall dialysis rate. Moreover, things may become more complicated when together with f also viscosity changes, i.e. when low values of f combine with high protein concentrations. An experiment like the one above, done with serially diluted serum and ^{3}H-cortisol as the migrating ligand showed different dialysis rates for 8 and 20 rpm at all dilutions but the $(\Delta 1/U)^{-1}$ vs. f curve obtained had an y-intercept not significantly different from zero. This observation is interpreted, with help of eq.(VA-5) as a decrease of the diffusion coefficient D_p and an increase of the film thickness caused by the higher viscosity in the more concentrated solutions. These effects will make the last term of eq.(VA-4) a function of the serum concentration like the other term which contains f, so

that the complete right hand member decreases with increasing concentration and decreasing f.

Further support for the film diffusion concept as an explanation for the effect of agitation speed was found by means of dialysis rate measurements in a pseudo-infinite volume set-up (appendix IIA, eq.(IIA-3)). Dialysis of buffer against 1% bovine serum albumin (BSA) yields for T4 tracer a situation where free T4 at the albumin side is virtually zero as compared with the buffer compartment. It can be understood that the cell permeability factor, accounting for film diffusion, is the same as the one that can be derived from (II-23), with the exception of the factor 2 in the film diffusion term. The pseudo-infinite volume dialysis rate U_{ps} is this instance becomes:

$$U_{ps} = -(1/t)\ \ln\ (c_1/(c_1 + c_2)) \qquad \text{(VA-5)}$$

$$\doteq (A/V)\ f\left(\frac{h}{D_m} + \frac{\delta f}{D_\ell}\right)^{-1}$$

The reciprocal is:

$$1/U_{ps} = (1/f).(V/A).\left(\frac{\delta f}{D_\ell} + \frac{h}{D_m}\right) \qquad \text{(VA-6)}$$

Subtraction from (VA-1) and substituting f = 1 gives:

$$1/U_{sy} - 1/U_{ps} = (V/2A)\ \frac{h}{D_m} \qquad \text{(VA-7)}$$

Where U_{sy} stands for symmetric dialysis rate. For Diachema membrane a pseudo-infinite volume dialysis rate of $1.339 \pm 0.0441\ h^{-1}$ and a symmetric dialysis rate of 1.724 ± 0.074 was obtained. Thus, a value of 6.00 ± 1.58 was obtained for $2AD_m/hV$ of which the confidence interval encloses the value of 4.25 found by direct assay using a reference solution. With complete membrane diffusion rate control, U_{sy} would have been twice U_{ps}. The fact that it is less, demonstrates prevailance of partial film diffusion control.

Now that the essentials of two dialysis methods for free hormone assay have been treated theoretically and by subsequent experimental verification of this theoretical approach it should be possible to tell whether the introduction of symmetric dialysis must be considered as a valuable contribution to the field of free hormone assay. A way to do so is by assesment of the problems of equilibrium dialysis (including the improvements proposed in this study) it solves, set against new problems it entails. Of course such a balance will look different depending on the type of free hormone assay considered.

From the purely practical point of view there is not much difference in the manipulations needed to carry out an assay by either equilibrium or symmetric dialysis if the Dianorm equipment or an equivalent thereof (Ch III.1) is employed. However, important differences arise in the acquisition from raw data of measurement data for further evaluation. For equilibrium dialysis with direct free hormone assay in the dialysate, the only concern is radioimmunoassay precision and sensitivity, but when using the indirect method, tracer recovery must be determined first, which necessitates assesment of fluid shift. With complete recovery, subsequent assays require measurements of radioactivity in dialysates only, but when this is not the case, each assay requires radioactivity measurements in both dialysate and dialysand which necessitates assesment of fluid shift in order to be able to determine the free hormone fraction according to methods given in Ch V. None such measures are required in symmetric dialysis.

With regard to the effect of radiochemical impurities of the used tracer hormone, it was shown both in theory and practice (Ch V.4) that assay by means of equilibrium dialysis is much stronger affected than symmetric dialysis during the same time of the same sample using the same tracer preparation. Besides purification of

the tracer, problems arising from contamination can be counteracted in equilibrium dialysis only by dilution of the sample before assay. This increases the proportion of intact tracer hormone in the dialysate relative to impurity. However, at the same time the already existing dilution inherent in equilibrium dialysis (Ch I.5) is increased, thus adding to the difficulty involved in correcting back to the undiluted states(Ch I.4, Ch IV). In symmetric dialysis there is the additional possibility of prolonging the dialysis time, which is exactly as effective in reducing the artefact (Ch V.4), but does not create problems involved in correction of the measurement results back to the undiluted state. Thus, symmetric dialysis is performed on diluted samples only when impractical long dialysis periods would be required for assay in diluted form.

The corrections for dilution by procedures given in Ch III 1 2 and Ch IV.2 are based on a model for hormone protein interaction which does not account for cooperative effects. Also the possibility that the free hormone concentrations in dialysand and dialysate are not identical as a consequence of Donnan effect is not included. However, if these effects are absent, the measurement data of equilibrium dialysis yields well-defined estimates of free hormone fractions according to the procedures mentioned and it is even possible to obtain true free fractions for diluted samples. Symmetric dialysis, in contrast, gives results in terms of a dialysis rate, which can be translated into the free hormone fraction when the corresponding cell permeability factor is known. The mathematical model for symmetric dialysis in its simplest form assumes that this factor is a constant. This was found to hold except for the region of high free hormone fractions (Ch V.3), although a case was encountered where this constancy was preserved up to f = 1.

Assays in the range of constant cell permeability factor pose the least problems. They require assessment of the dialysis rate of one reference solution of which the free fraction is known and is located in the linear range of the dialysis rate vs.free hormone fraction curve. The curvilinearity of the upper part of such a standard curve was found to be adequately explained by the film

diffusion concept (Ch II.4.2) in which a role in determining the rate of tracer transfer is ascribed to its diffusion in the liquid films adhering to the membrane in addition to diffusion through the membrane. In this concept the cell permeability factor becomes a function of free hormone fraction itself, but it is noticeable at higher values of f only. Still, the only way to determine free hormone fractions in the range of non-constancy of the cell permeability factor would be by construction a standard curve by means of several reference samples with known free fraction (Ch V·3,3). In this respect equilibrium dialysis is the most straightforward method since assay standards are not required for this method.
It also should be mentioned that in symmetric dialysis, at least in principle, the possibility exists that association or dissociation rates of the tracer-protein complexes influence the dialysis rate (Ch II.4,3). Although such cases have not been encountered, their possible occurence should be kept in mind. Obviously, this too is irrelevant to equilibrium dialysis.
Summarising it may be stated that for assay of undiluted samples, symmetric dialysis is superior to equilibrium dialysis using the indirect method in the following respects:

a. acquisition of measurement data
b. susceptibility to tracer impurities
c. precision (Ch V.4.1)
d. absence of any effect related to dilution
e. absence of Donnan effect

With regard to one, by no means unimportant aspect, equilibrium dialysis is superior in that no standards are needed to obtain an estimate of the free hormone fraction. When direct assay of free hormone in equilibrium dialysates is compared with symmetric dialysis, only points d. and e. are relevant, while for point c. no general statement can be made. When such comparison is made for assay of diluted sera, points d. and possible e. lose their relevance.
According to the above considerations, the choice of methodology for the free hormone assays dealt with in this study would be as follows:
In free T4 and free T3 assays the major problem is tracer contamination because of the low free fractions of these hormones.

By virtue of the same fact, the free fractions are directly proportioned to the dialysis rate so that one single standard suffices for the assay. Therefore, symmetric dialysis is the method of choice in these cases. Since dialysis of undiluted serum would require impractical long dialysis times for routine assays, especially in the case of free T4, these assays are performed on diluted serum. When this approach is followed, the precise effect of dilution (Ch I.6, Ch V.4.2) remains to be established, especially with regard to the buffer used for diluting. Free T4 and free T3 measurement by direct assay in equilibrium dialysates is also very elegant, since it does not posses most of the drawbacks of the indirect method. Symmetric dialysis has as the only advantages its possibility for measurement in the true undiluted state, and the fact that it does not rely on the availability of highly sensitive T4 and T3 radioimmunoassays. For the assay of diluted samples, both methods are equally well suited (Ch V.5).

In free cortisol assay the major problem is, as concluded from Ch I.2, IA.2.3 and Ch IV.4, the effect of dilution inherent in equilibrium dialysis, regardless of whether a direct or indirect assay is applied. Whereas for free T4 and T3 the dilution effect is simple and well-defined when the proper electrolyte balance is maintained, this is not so for free cortisol, so that really accurate measurements of free cortisol in undiluted serum are possible only by symmetric dialysis. Since under proper experimental conditions a direct proportionality of free hormone fraction and dialysis rate for twice diluted serum up to pure buffer (f = 1). was found, this method must be the method of choice for this assay. If it would appear from further study that a well-defined relation between the free fraction in undiluted and that in e.g. twice diluted serum exists, the possibility of direct equilibrium dialysis assay in the diluted state should be taken in consideration for practical reasons.
Free aldosterone finally, can be adequately assessed by equilibrium dialysis, since the very high free hormone fraction makes the assay relatively insensitive to tracer contamination and the effect of dilution is well defined and simple (linear dilution

effect, Ch I.2.6, Ch IV.3). If suitable materials are used so that tracer is completely recovered, the simple method of radioactivity measurement in dialysate only, can be used. Symmetric dialysis is not recommended for this purpose. The high free fraction will most likely fall in the non-linear part of the standard curve, where also viscosity plays a role. A standard curve would be required consisting of standard solutions with the same viscosity as the unknown. Furthermore, the very short dialysis time reduces accuracy and precision of dialysis rate estimates.

The range of non-constancy of the cell permeability factor where tracer transport in the liquid films, adhering to the membrane, influences the dialysis rate might prove interesting from another point of view. As it may be expected that in the slowly flowing interstitial fluid surrounding tissue cells a relatively stationnary layer at the interface with the cells will exist, where hormone transport towards the cell must take place by diffusion only, the dialysis cell may serve as in vitro model system for the study of hormone transport into cells. Of course the symmetric arrangement would be abandoned in such a system.

As final conclusions about the two dialysis methods it may be stated that the possibilities of equilibrium dialysis are not completely exhausted yet. Although symmetric dialysis may not be the method of choice for any possible type of free hormone assay, it is unique in that it permits free hormone assays in the true undiluted state, with relatively low interference from tracer defectiveness. When applied for this purpose, it may disclose hitherto unknown facts about free hormone physiology especially in those instances where the effect of dilution is poorly defined. It was felt that development in the field of free hormone study has been more or less stationary for the last ten years. The problems of equilibrium dialysis have remained almost the same since the technique was introduced in 1959 by Slaunwhite and Sandberg. The recent development of free thyroid hormone assays by direct assay in equilibrium dialysates as introduced by Ekins has stimulated renewed interest. It is hoped that by the introduction of symmetric dialysis and the improvements proposed for equilibrium dialysis an additional stimulus for future interest and research on free hormones is provided.

Summary

The concentration of free, non-protein bound iodothyronine or steroid hormone in blood is generally assumed to represent the biological action of these hormones more accurately than their total levels. Free hormones are assessed most often by equilibrium dialysis of serum to which a tracer amount of radioactive hormone has been added, against a certain volume of buffer. Since the dialysis membrane that is placed between serum and buffer compartments is permeable to free hormone only, at final equilibrium the concentrations of free hormone and free tracer will have become equal on both sides of the membrane. As the serum compartment (dialysand) contains both bound and free hormone, whereas the buffer compartment (dialysate) contains only free, the distribution of tracer hormone over the two compartments permits calculation of the fraction of the hormone that is free, which gives the free hormone concentration after multiplication with the total hormone concentration. More recently, methods in which free hormone is directly assessed by radioimmunoassay in the dialysate, have been described. The indirect assay, using tracer hormone to determine the free hormone fraction, may suffer from artefacts arising from tracer contamination, that may be counteracted by sample dilution. This however introduces a new problem since the effect of dilution on free hormone is in general poorly defined. Another type of dilution involved is shift of buffer towards the serum compartment due to osmotic pressure. Also the effect that diffusion of some hormone out of the serum compartment, so that its total concentration there is reduced, must have on the hormone-protein equilibria involved, has not been assessed until now. One objective of this thesis is obtaining more insight in these problems so that they can be managed more adequately. As the reach of this approach is restricted to particular (though frequently encountered) conditions, a new method was developed which is based on measurement of the rate at which tracer hormone migrates from one serum compartment through the membrane into the other compartment which contains the same serum sample ("Symmetric dialysis"). As identical samples are present on both sides of the

membrane, dilution effects, other than those introduced deliberately for the sake of assay of diluted samples, are completely absent. By virtue of the fact that the tracer distribution with time approaches equal partionning over the two compartments, the method is much less susceptible to interference by tracer impurities. So, the other objective of this thesis is to obtain, by means of theoretical and experimental study, an impression of the potential of this method, and how equilibrium dialysis and symmetric dialysis may complement each other.

Chapter I starts with introduction of a mathematical model for interaction of a hormone with multiple binding sites. With this model the effects of changing total hormone concentration and dilution on the free hormone concentration are studied both by theoretical analysis and simulation of actual hormone binding systems. Especially the effect of dilution receives much attention since in the final paragraph it is proven from the model that application of equilibrium dialysis has the same effect on the free hormone concentration as mere dilution. Shifts of fluid within the dialysis system have no effect. It is found that under certain conditions the effect of dilution on free hormone can be described by a simple relation.

In chapter II the first objective is to examine which of four different types of dialysis arrangements is suitable for free hormone assay by way of dialysis rate assessment. Therefore first the time course of a general type of dialysis process in which arbitrary amounts of ligand binding protein are present on both sides of the membrane, and of which the four types mentioned are particular cases, is described. It is found that symmetric dialysis is by far best-suited for the propose. As the general model was derived with the assumption that only the diffusion of tracer within the membrane determines the overall rate of tracer diffusion, the mathematical model for symmetric dialysis was extended to include other possible rate-limiting factors also. The film diffusion concept by Nernst was adapted to the model to account for the possibility that diffusion in the stationary liquid films

adhering to the membrane will influence the rate of tracer transport. Also the possibility that slow dissociation or association of hormone-protein complexes influence the dialysis rate, was incorporated into the model.

Chapter III deals, in addition to the basic techniques as employed in the ensuing chapters, with some straightforward procedures for setting up routine free hormone assays, both for equilibrium and symmetric dialysis.

In chapter IV on equilibrium dialysis first some calculation procedures for estimation of the free hormone concentration and fraction are derived by using the theory of chapter I. This is done for the direct and the indirect method. Calculation procedures from the literature from the latter method are critically reviewed, also against the setting of the theory of the effect of dilution from chapter I and the derived calculation procedures. These procedures compare favourably with existing ones in that a correction for dilution is incorporated which has more general validity. Moreover, one of these two procedures, which may be applied in all cases where tracer is completely recovered, provides a considerable practical advantage since it is based on measurement of radioactivity in the dialysate only. Subsequently the concepts underlying these procedures as well as the procedures themselves are experimentally verified by means of free aldosterone and free cortisol assays.

In chapter V, which deals with symmetric dialysis, it is examined whether the time course of tracer distribution follows accurately the theoretical model. This implies that an unequivocal estimate of the dialysis rate, which ideally is directly proportional to the free hormone fraction, can be readily obtained. The relation between dialysis rate and free hormone fraction is further studied in view of the film diffusion concept, which is found to explain adequately the effects of changing the agitation efficiency on the linearity of the relation in the upper range of free hormone fractions. Effects of tracer contamination are studied by means

of free thyroxine assays by equilibrium and symmetric dialysis. In the latter case tracer contamination also under unfavourable conditions can be managed quite adequately by making use of the fact that both precision and accuracy can be optimised by choosing the appropriate length of dialysis time. Equilibrium dialysis by the indirect method in contrast, will necessitate extensive tracer purification, such as described in the litterature. Finally it is shown that symmetric dialysis is at least as suitable for the assay of free thyroid hormones as existing techniques, by means of brief studies on free thyroid hormones during the first trimester of pregnancy, in TBG deficiency and in thyroid disease.

It is concluded that symmetric dialysis is promising, especially for free hormone assays in the true undiluted state and for hormones of which a relatively small proportion is in the unbound form. For hormones less avidly bound than cortisol, equilibrium dialysis will most often remain the method of choice, provided the proposed modifications for the evaluation of measurement data are included.

Algemeen wordt aangenomen dat de concentratie van het vrije, niet aan eiwit gebonden jood-thyronine- en steroïdhormoon in het bloed, beter de biologische activiteit van deze hormone weergeeft dan hun totale spiegels. Vrij hormoon wordt meestal bepaald door middel van evenwichtsdialyse van serum, waaraan een zeer kleine hoeveelheid radioactief gemerkt hormoon is toegevoegd, tegen een bepaalde hoeveelheid buffer. Daar het dialysemembraan, dat zich tussen serum- en buffercompartiment bevindt, uitsluitend vrij hormoon doorlaat, zullen de concentraties van vrij hormoon - zowel het radioactieve als het niet-radioactieve - aan beide zijden van het membraan gelijk zijn wanneer evenwicht is bereikt. Doordat het serumcompartiment (dialysand) zowel gebonden als vrij hormoon bevat, terwijl in het buffercompartiment (dialysate) zich alleen vrij hormoon bevindt, maakt de verdeling van radioactief hormoon over beide compartimenten het mogelijk de vrije hormoon-fractie te berekenen. De concentratie van het vrije hormoon volgt dan door vermenigvuldiging van de vrije fractie met de totale hormoonconcentratie. Methoden ter directe bepaling van vrij hormoon in evenwichtsdialysaten middels radioimmunoassay zijn van recentere datum. De indirecte bepaling, waarbij gebruik gemaakt wordt van radioactief ("tracer") hormoon kan gestoord worden door de aanwezigheid van radioactieve verontreinigingen van de gebruikte tracer, hetgeen enigermate tegengegaan wordt door verdunning van het te analyseren monster. Deze maatregel schept vaak nieuwe problemen daar het effect van verdunning op de vrije hormoonconcentratie veelal onvoldoende bekend is. Verdunning kan ook optreden door verplaatsing van oplosmiddel naar het serumcompartiment als gevolg van verschil in osmotische druk met de buffer. Ook het effect van daling van de totale hormoonconcentratie in het serumcompartiment, tengevolge van het feit dat een zekere hoeveelheid hormoon naar het buffercompartiment diffundeert, werd tot nu toe niet onderzocht. Een van de doelstellingen van dit proefschrift is het verkrijgen van meer inzicht in deze problemen zodat deze beter onder controle kunnen worden gekregen. Daar de mogelijkheden van deze benadering beperkt blijven tot een aantal bijzondere, hoewel vaak voorkomende, gevallen, werd een

nieuwe methode ontwikkeld welke gebaseerd is op meting van de snelheid waarmee tracer hormoon zich verplaatst van het ene serum compartiment door het membraan naar het andere compartiment dat hetzelfde monster bevat ("symmetrische dialyse"). Daar identieke oplossingen aanwezig zijn aan beide zijden van het membraan, kunnen verdunningseffecten, anders dan die met opzet uitgevoerd voor onderzoek van een verdund monster, niet optreden. Dank zij het feit dat de verdeling van tracer over beide compartimenten met het verloop van de tijd gelijk wordt, is de methode veel minder gevoelig voor storingen door tracer verontreiniging. Daarom is de tweede doelstelling van dit proefschrift het onderzoeken van de mogelijkheden van deze methode en vergelijken met evenwichtsdialyse zodat een indruk verkregen wordt hoe de twee methoden elkaar kunnen aanvullen.

Hoofdstuk I begint met de introductie van een wiskundig model dat de interactie van een hormoon met een willekeurig aantal typen bindingsplaatsen beschrijft. Met behulp van dit model worden de effecten van verandering van totale hormoonconcentratie en verdunning op de vrije hormoonconcentratie bestudeerd, zowel door middel van theoretische analyse van het model als door simulatie van bestaande hormoon-bindend eiwit systemen. Vooral wordt aandacht besteed aan het effect van verdunning omdat in de laatste paragraaf wordt bewezen dat op grond van dit model het toepassen van evenwichtsdialyse hetzelfde effect heeft op de vrije hormoonconcentratie als verdunnen op zich. Verschuivingen van vloeistof binnen het dialysesysteem hebben geen effect. Tevens wordt getoond dat onder bepaalde voorwaarden het effect van verdunnen door een eenvoudige relatie beschreven kan worden.

In hoofdstuk II wordt als eerste onderzocht welke van vier verschillende dialyse opstellingen geschikt is voor het bepalen van vrij hormoon door middel van meting van de dialysesnelheid. Voor dit doel wordt eerst het tijdsverloop van een algemeen type dialyse proces , waarbij willekeurige hoeveelheden bindend eiwit aan weerszijden van het membraan worden aangenomen, afgeleid. De vier genoemde opstellingen zijn dan als bijzondere gevallen hiervan te

beschouwen. Gevonden wordt, dat van deze vier de symmetrische dialyse opstelling verreweg het meest geschikt is. Daar het algemene model afgeleid werd met de aanname dat uitsluitend de diffusie van de tracer in het membraan de waargenomen dialysesnelheid bepaalt, wordt het model voor symmetrische dialyse uitgebreid met de mogelijkheid dat andere processen mede snelheidsbepalend zijn. Het film-diffusie principe van Nernst wordt aangepast zodanig dat de mogelijkheid, dat diffusie van tracer in de stationaire vloeistoffilms aan het grensvlak van het membraan de snelheid van het transport van tracer beïnvloedt, in het model kan worden opgenomen. Tevens wordt het model uitgebreid met de mogelijkheid van beïnvloeding door dissociatie en associatiesnelheden van de aanwezige hormoon-eiwit-complexen.

Hoofdstuk III behandelt naast de in de navolgende hoofdstukken toegepaste technieken, enkele eenvoudige procedures voor het opzetten van routinematige vrije hormoonbepalingen, zowel met behulp van evenwichts- als symmetrische dialyse.

In hoofdstuk IV waarin de evenwichtsdialyse behandeld wordt, worden eerst enkele berekeningsvoorschriften ter bepaling van de vrije hormoonconcentratie en -fractie afgeleid met behulp van de theorie uit hoofdstuk I, zowel voor de directe als de indirecte methode. Berekeningsvoorschriften uit de literatuur met betrekking tot deze laatste methode worden kritisch bezien tegen de achtergrond van de theorie betreffende het effect van verdunning uit hoofdstuk I en de hier afgeleide voorschriften. Deze laatsten hebben als voordeel boven de bestaande procedures dat de wijze waarop verdunning in rekening wordt gebracht een meer algemene geldigheid heeft. Wanneer alle toegevoegde tracer in oplossing teruggevonden wordt, mag een berekening worden toegepast die als extra voordeel biedt dat hiervoor alleen een radioactiviteitsmeting in het dialysaat benodigd is. Genoemde berekeningsmethoden, alsmede de eraan ten grondslag liggende principes, worden vervolgens experimenteel getoetst door middel van bepalingen van vrij aldosteron en vrij cortisol.

In hoofdstuk V, handelend over symmetrische dialye, wordt eerst

experimenteel onderzocht of het verloop in de tijd van de tracerverdeling het theoretische model nauwkeurig volgt. Dit houdt in dat de dialysesnelheid, welke idealiter recht evenredig is met de vrije hormoonfractie, op ondubbelzinnige wijze kan worden bepaald. De relatie tussen dialysesnelheid en vrije hormoonfractie wordt verder bestudeerd in verband met het filmdiffusie model, waarbij blijkt dat met dit principe een toereikende verklaring kan worden gegeven voor de waargenomen invloed van de intensiteit waarmee de oplossingen gemengd worden tijdens dialyse op de lineairiteit van de relatie bij hoge vrije fractie. De invloed van tracerverontreiniging wordt bestudeerd aan de hand van bepalingen van vrije thyroxine door middel van evenwichts- en symmetrische dialyse. In het laatste geval blijkt dat de effecten van tracerverontreiniging ook onder ongunstige omstandigheden goed in de hand kunnen worden gehouden dank zij de mogelijkheid tot optimalisering van de meetnauwkeurigheid en minimalisering van de invloed van storing door radio-chemische verontreiniging door keuze van de juiste dialysetijd. Bij evenwichtsdialyse volgens de indirecte methode, daarentegen, is uitgebreide zuivering van de tracer noodzakelijk, zoals in de literatuur is beschreven. Aan het eind van het hoofdstuk wordt getoond dat symmetrische dialyse tenminste even geschikt is voor de bepaling van de vrije schildklierhormonen als bestaande methoden. Dit wordt gedaan aan de hand van korte studies van vrije schildklierhormoon spiegels gedurende het eerste trimester van de zwangerschap, bij thyroxine-bindend globuline deficientie en bij schildklierpathologie.

De uiteindelijke conclusie luidt, dat symmetrische dialyse een veelbelovende methode is, in het bijzonder voor metingen van vrij hormoon in werkelijk onverdunde toestand van het serum en voor hormonen waarvan slechts een zeer kleine fractie in de vrije vorm aanwezig is. Voor hormonen die minder sterk gebonden zijn dan cortisol, zal vaak nog de evenwichtsdialyse de aangewezen methode zijn, indien de hier voorgestelde verbeteringen wat betreft de behandeling van de meetgegevens worden toegepast.

References.

Avruskin T.W., Misuma T, Shenkman L, Sau L, Hollander C.S. (1976): Measurement of free and total serum T3 and T4 in pregnant subjects and in neonates. Am.J.Med.Sci. 271, 309.

Cavalieri R.R., Ingbar S.H. (1975): Methods for measuring the thyroxine-binding proteins and free thyroid hormone concentration in serum. In: Methods in Enzymology vol. XXXVI Hormone action, part A, Steroid Hormones. O'Mally, Hardman eds. Academic Press New York 1975, p. 126.

Christensen L.K. (1959): A method for the determination of free, non-protein bound thyroxine in serum. Scand.J.Clin.Lab.Invest. 11, 326.

Clerico A., Del Chicca M.G., Zuchelli G., Mariani F., Materazzi F. (1978): Radioimmunological determination of free (diffusible) cortisol in an equilibrium dialysis system. J.Nucl.Med.All.Sci. 21,5.

Dixon P.F. (1968): The kinetics of the exchange between transcortin bound and unbound cortisol in plasma. J.Endocr. 40, 457.

Ekins R.P., Ellis S.M. (1975): The radioimmunoassay of free thyroid hormones in serum. In: Thyroid Research, Proceedings of the Seventh International Thyroid Conference Boston, 1975. Excerpta Medica Amsterdam-Oxford 1976, p.597.

Ekins R.P. (1979): Methods for the measurement of free thyroid hormones. In: Free thyroid hormones, International Symposium, Venice 1978, Excerpta Medica Amsterdam 1979, p.72.

Evered D.C., Yeo P.D.B., Gomez-Pan A., Bates D., Lewis M. (1977): Direct measurement of free T4 and free T3 in patients with altered thyroid hormone binding protein capacity. 8th Annual Meeting of the European Thyroid Association, Lyon 1977. Annales d'Endocrinologie 38, 79A.

Exalto N. (1979): Early pregnancy. Thesis, University of Nijmegen.

Feldman H.A. (1972): Mathematical theory of complex ligand-binding systems at equilibrium. Anal.Biochem. 48, 317.

Helfferich F. (1962): Ion exchange, McGraw-Hill, New York 1962, pp.253-352.

Herrmann J., Krüskemper H.L. (1971): Freies Thyroxin im Serum. Z.Klin.Chem.Klin.Biochem. 9, 320.

Hillier A.P. (1975): Thyroxine dissociation in human plasma: Measurement of its rate by a continuous-flow dialysis method. Acta Endocrinol. 78, 32.

Hirsch R.F. (1977): Analysis of variance in analytical chemistry. Anal.Chem. 49. 691A.

Ingbar S.H., Braverman L.E., Dawber N.A., Lee G.Y. (1965): A new method for measuring the free thyroid hormone in human serum and an analysis of the factors that influence its concentration. J.Clin.Invest. 44, 1679.

Irvine C.H.G. (1974): Measurement of free thyroxine in human serum by a Sephadex binding method. J.Clin.Endocrinol.Metab. 38, 655.

Kley H.K., Bartmann E., Krüskemper H.L. (1977A): The measurement of free cortisol, free testosterone and free estradiol-17β: A comparison of five different methods. J.Clin.Chem.Clin.Biochem. 15, 465.

Kley H.K., Bartmann E., Krüskemper H.L. (1977B): A simple and rapid method to measure non-protein-bound fractions of cortisol, testosterone and estradiol by equilibrium dialysis: Comparison with centrifugal filtration. Acta Endocrinol. 85, 209.

Ködding R., von zur Mühlen A. (1975): Untersuchungen zur Herstellung von ^{125}J-markierten Triiodthyronin und Thyroxin mit hohen Spezifischer Radioaktivität. Z.Klin.Chem.Klin.Biochem. 13, 563.

Konno N. (1976): Serum thyrotropin response to thyrotropin releasing hormone and free thyroid hormone indices in patients with familiar thyroxine binding globulin deficiency. Endocrinol.Jpn. 23, 313.

Krenning E.P., Docter R., Bernard H.F., Visser T.J., Hennemann G. (1979): The essential role of albumin in the active transport of thyroid hormones into primary cultured rat hepatocytes. FEBS letters 107 (1), 227.

Kristensen B.O., Weeke J. (1977): Propranolol-induced increments in total and free serum thyroxine in patients with essential hypertension. Clin.Pharmacol.Ther. 22, 864.

Lutz R.A., Märki H.H., Weder H.G. (1977): Androgenbinding in human plasma and its physiological significance. J.Clin.Chem.Clin.Biochem. 15, 57.

de Man A.J.M., Benraad Th.J. (1977): Aldosterone secretion rate: Radioimmunoassay versus double isotope derivative assay. Clin. Chim.Acta 79, 489.

de Man A.J.M., Hofman J.A., Hendriks Th., Rosmalen F.M.A., Ross H.A., Benraad Th.J. (1979): A direct radioimmunoassay for plasma aldosterone: Significance of endogenous cortisol. Neth.J.Med., in press.

Meyer C.J., Layne D.S., Tait I.E., Pincus G. (1961): The binding of aldosterone to plasma proteins in normal, pregnant and steroid-treated women. J.Clin.Invest. 40, 1663.

Mills I.H. (1962): Transport and metabolism of steroid. Brit.Med. Bull. 18, 127.

Moll G.W., Rosenfield R.L. (1977): A flow dialysis technique for the measurement of free testosterone in human plasma under physiological conditions. Biochem.Med. 18, 344.

Moll G.W., Rosenfield R.L. (1978): Some limitations of using equilibrium dialysis to study human serum albumin-testosterone interaction. J.Clin.Endocrinol.Metab. 46, 501.

Odstrchel G., Herte W., Ward F.B., Travis K., Lindner R.E., Mason R.D. (1978): New concepts for the assay of unbound thyroxine (FT4) and thyroxine binding globulin (TBG). In: Radioimmunoassays and related procedures in medicine, vol. II, I.A.E.A.Vienna, p.369.

Olsen T. (1979): Free T4, free T3 and free reverse T3 in dialysates of serum: The influence of electrolytes and pH with special reference to the physiological range. Scand.J.Clin.Lab.Invest. 39, 53.

Oppenheimer J.H., Squef R., Surks M.I., Hauer H. (1963): Binding of thyroxine by serum proteins evaluated by equilibrium dialysis and electrophoretic techniques. Alterations in non-thyroidal illness. J.Clin.Invest. 42, 1769.

Oppenheimer J.H., Surks M.I. (1964): Determination of free thyroxine in serum: A theoretical and experimental analysis. J.Clin.Endocrinol.Metab. 24, 785.

Osatanondh R., Tulchinsky D., Chopra I.J. (1976): Total and free thyroxine and triiodothyronine in normal and complicated pregnancy. J.Clin.Endocrinol.Metab. 42, 98.

Pedersen K.O. (1974). A systematic study of variables affecting protein binding of thyroxine and triiodothyronine in serum. Scand. J.Clin.Lab.Invest. 34, 247.

Pedersen P.V., Crooks M.J., Brown K.F. (1977): Method of obtaining drug-macromolecule binding parameters directly from dynamic dialysis data. J.Pharm.Sci. 66, 1458.

Premachandra B.N., Gossain V.V., Perlstein I.B. (1976): Increased free thyroxine in a euthyroid patient with thyroxine binding-globulin deficiency. J.Clin.Endocrinol.Metab. 42, 309.

Prince H.P., Ramsden D.B. (1977): A new theoretical description of the binding of thyroid hormones by serum proteins. Clin.Endocrinol. 7, 307.

Robbins J., Rall J.E. (1967): The iodine-containing hormones. In: Hormones in blood, Gray, Bacharach eds., 2nd ed. vol.1, Academic Press New York, p.383.

Robbins J. (1974): Structure and function of thyroxine transport proteins. In: Thyroid Hormone Metabolism. Harland, Orr, eds. Academic Press London 1975, p.1.

Romelli P.B., Pennisi F., Vancheri L. (1979): Measurement of free thyroid hormones in serum by column adsorption chromatography and radioimmunoassay. J.Endocrinol.Invest. 2, 25.

Ross H.A. (1978): A dialysis rate method for the measurement of free iodothyronine and steroid hormones in blood. Experientia 34, 538.

Ross H.A. (1979): A dialysis rate method for the measurement of free thyroid hormones in serum. J.Endocr. 80, 22P.

Schussler G.C., Plager J.E. (1967): Effect of preliminary purification of ^{125}I-thyroxine on the determination of free thyroxine in serum. J.Clin.Endocrinol.Metab. 27, 242.

Slaunwhite W.R., Sandberg A.A. (1959): Transcortin: A corticosteroid-binding protein of plasma. J.Clin.Invest. 38, 384.

Smals A.G.H., Ross H.A., Benraad Th.J., Kloppenborg P.W.C. (1979): Serum free T3 and T4 levels in TBG deficiency measured by symmetric dialysis. In preparation.

Spaulding S.W., Gregerman R.I. (1972): Free thyroxine in serum by equilibrium dialysis: Effects of dilution, specific ions and inhibitors of binding. J.Clin.Endocrinol.Metab. 34, 974.

Sterling K., Brenner M.A. (1966): Free thyroxine in serum: simplified measurement with the aid of magnesium chloride precipitation. J.Clin.Invest. 45, 153.

Sutherland R.L., Brandon M.R., Simpson-Morgan M.W. (1975): Effect of ionic strength and ionic composition of assay buffers on the interaction of thyroxine with plasma proteins. J.Endocr. 66, 319.

Tait I.F., Burstein S. (1964): In vivo studies of steroid. In: The hormones, vol.V. Pincus S., Thimann K.V., Astwood E.B. eds. Academic Press New York-London, p.441.

Tanaka S., Starr P. (1959): A euthyroid man without thyroxine-binding globulin. J.Clin.Endocrinol.Metab. 19, 485.

Thijssen J.H.M., der Kinderen P.J., Ross H.A., Tertoolen I.F.W., Visser J.W.E. (1979): Results of a comparative study of free thyroxine determinations. J.Endocr. 80, 22P.

Uchimura H., Nagataki S., Tabuchi T., Mizuno M., Ingbar S.H. (1976): Measurements of free thyroxine: Comparison of percent of free thyroxine in diluted and undiluted seta. J.Clin.Endocrinol.Metab. 42, 561.

Vermeulen A., Stoica T., Verdonck L. (1971): The apparent free testosterone concentration, an index of androgenicity. J.Clin. Endocrinol.Metab. 33, 759.

Weeke J., Orskov H. (1979): Measurement of free thyroid hormones by dialysis and radio-immunoassay. In: Free thyroid hormones, International symposium, Venice 1978. Excerpta Medica Amsterdam p.137.

Westphal U. (1971): Steroid-protein interactions. Springer Verlag, New York.

Westphal U. (1977): Zur bindung von Steroidhormonen an Serumproteine. Klin.Wochenschr. 55, 877.

Wosilait W.D. (1977): A theoretical analysis of the distribution of thyroxine among sites on thyroid binding globulin, thyroid binding prealbumin and serum albumin. Res.Comm.Chem.Pathol.Pharmacol. 16, 541.

Yeo P.P.B., Lewis M., Evered D.C. (1977): Radioimmunoassay of free thyroid hormone concentrations in the investigation of thyroid disease. Clin.Endocrinol. 6, 159.

Zager P.G., Burtis W.J., Luetscher A.J., Dowdy A.J., Sood S. (1976): Increased plasma protein binding and lower metabolic clearance rate of aldosterone in plasma of low cortisol concentration. J.Clin.Endocrinol,Metab. 42, 207.

A	membrane area (cm^2)
B_1, $\hat{B}_1$	concentration of hormone or tracer hormone, bound to binding sites of order 1.
b_1	fraction of total hormone bound to binding sites of order 1.
b	fraction of total hormone bound ($b = \Sigma\ b_1$)
C	integration constant
$\underline{C}$	amount of radioactive tracer hormone (cpm)
c_0	Equilibrium dialysis: concentration of labelled hormone (cpm/ml) in the original sample, before assay or dilution. Symmetric dialysis: Initial concentration of labelled hormone (cpm/ml) in the compartment where it is added at the start of the process (at $t = 0$, $c_1 = c_0$).
c_1	Equilibrium dialysis: concentration of labelled hormone in dialysand (serum compartment) at equilibrium. Symmetric dialysis: concentration of labelled hormone in compartment 1, where tracer was added initially.
c_2	Equilibrium dialysis: concentration of labelled hormone in the dialysate (buffer compartment) at equilibrium. Symmetric dialysis: concentration of labelled hormone in compartment 2 into which tracer is diffusing after its addition to compartment 1.

C.B.G.	corticosteroid binding globulin (transcortin)
cell permeability factor cell permeability constant	The ratio of dialysis rate c and free hormone fraction f, which is ideally a constant and depends only on membrane area, permeability and sample volume (for symmetric dialysis: $2AD_m/hV$). More generally, at high f it becomes also a function of f, agitation efficiency and solution viscosity as a consequence of the film diffusion effect.
cortisol	hydrocortisone (M.W. 362.5)
cpm	counts per minute.
C.V.	coefficient of variation (S.D./mean, %)
D	diffusion coefficient of (tracer) hormone in a membrane divided by membrane thickness h ($cm.sec^{-1}$)
D_m	diffusion coefficient of (tracer) hormone in a membrane ($cm^2.\ sec^{-1}$)
D_ℓ	diffusion coefficient of (tracer) hormone (bound + free) in solution ($cm^2.sec^{-1}$)
D'	cell permeability constant = AD_m/hV (sec^{-1}), in this study usually expressed in h^{-1}
D_1	diffusion coefficient in solution of the complex of (tracer) hormone with binding protein carrying binding sites of order 1 ($cm^2.sec^{-1}$)
D_f , D_h	diffusion coefficient in solution of unbound hormone

dialysand	solution volume in dialysis compartment containing binding macromolecules
dialysate	solution volume in dialysis compartment containing molecules, for which the membrane is permeable, only.
DF, DfT4	Notation used by Oppenheimer (1963, 1964) for dialysable fraction of hormone (T4): the amount of dialysable tracer hormone (c_2V_t) in both dialysis compartments divided by the total amount of tracer present ($c_1V_1 + c_2V_2$).
DPH	diphenylhydantoine
δ	Thickness of diffusion layer (film) adhering at the membrane surface.
ε	amount of non-hormonal radioactivity present as contamination in tracer preparations (cpm)
ε'	fractional contamination of tracer
F	concentration of free, non-protein bound hormone
$\hat{F}$	concentration of free, non-protein bound tracer hormone
F_0	concentration of free hormone in what is considered as the original (serum) sample before assay or dilution
F_0'	estimate of F_0, involving correction for dilution according to the assumption of a linear dilution effect.
f	fraction of total hormone that is present in the free form (i.e. free hormone fraction)

f_0	free hormone fraction in the original sample
f_0'	estimate of f_0 involving correction for dilution according to the assumption of a linear dilution effect.
f_0''	estimate of f_0 using a different correction for dilution
f_s	estimate of free hormone fraction according to c_2/c_1, Slaunwhite (1959)
f_r, f_x	free hormone fraction of reference solution and unknown respectively
FT4, FT3	concentrations of free T4 and T3
fT4%, fT3%	free T4 and T3 fractions expressed in %.
f/b	ratio of free to bound hormone concentrations
film diffusion concept	model for transport of solute towards a solid surface which replaces decreasing importance of convection and increasing importance of diffusion as a means of transport by a sharply defined layer (film) in which diffusion is the sole mode of transport and which is located between the well-stirred solution and the solid surface.
film diffusion effect	dependence of the dialysis rate not only on tracer diffusion in the membrane, but also on diffusion in the liquid films adhering to the membrane. It results in non-linearity of U as a function of f.
H	total hormone concentration

H_0	total hormone concentration in what is considered as the original sample before assay
H_1	bound + free hormone concentration in dialysand
H_2	the same in the dialysate
h	membrane thickness
H.S.A.	human serum albumin
I	isotope distribution, defined as the ratio $(c_1-c_2)/(c_1+c_2) = (c_1-c_2)/c_o$. For symmetric dialysis it is identical to Q_t.
J	flux, flow of material through unit cross-section per unit time (mol.sec $^{-1}$cm^{-2})
K	equilibrium (dissociation) constant (mol/l)
K'	equilibrium (association) constant (l/mol)
k	dissociation rate constant
k'	association rate constant
L	dilution factor
linear dilution effect	phenomenon of direct proportionality of free to bound (f/b) ratio with dilution.
P_1	concentration of binding sites of order 1.
P_{10}	concentration of binding sites of order 1 in the original sample before assay or dilution.

Q_t	ratio of concentration difference of free tracer hormone over the membrane at time t to that at t=0. Indicates how far a dialysis process still has to go towards equilibrium. In the case of symmetric dialysis it is equal to the ratio of total tracer differences, I.
r, r_s	correlation coefficient, according to Pearson and Spearman respectively.
S	flow of material through a surface ($mol.sec^{-1}$)
S.D.	standard deviation
S.E.M.	standard error of the mean ($S.D./n^{\frac{1}{2}}$)
TBG	thyroxine binding globulin
TBPA	thyroxine binding prealbumin
T4	thyroxine (M.W. 776.9)
T3	3, 5, 3' triiodothyronine (M.W. 651.0)
t	time
U	dialysis rate. Fractional rate of approach of dialysis equilibrium. In general $U = -(1/t) \ln Q_t$ and for symmetric dialysis $U = -(1/t) \ln I$. In the ideal case $U = 2 AD_m ft/hV$, i.e. a quantity directly proportional to the free hormone fraction f.
U_r, U_x	Dialysis rates of reference solution and unknown.
V_0	volume of original sample assayed by equilibrium dialysis.

V_0'	initial volume of dialysand in equilibrium dialysis, which contains a volume V_0 of the original sample.
V_d	initial volume of buffer or saline in the dialysate
V_1	dialysand volume at equilibrium
V_2	dialysate volume at equilibrium
V_t	total volume in the dialysis system ($V_t = V_0' + V_d = V_1 + V_2$)

Woorden van dank.

Allen die op directe of indirecte wijze hebben bijgedragen aan de tot standkoming van dit proefschrift wil ik op deze plaats van harte dank zeggen.
Enkele personen wil ik daarbij echter met name noemen.
Naast de onontbeerlijke morele steun verleende Trude ook daadwerkelijke hulp zowel door het door mij geschrevene geduldig in leesbare vorm te brengen, als bij het gereedmaken van het manuscript.
Belangrijk experimenteel werk werd gedaan door Ferry Hübner, terwijl op dit gebied eveneens waardevolle bijdragen werden geleverd door Oda Voesten, Joop Hövel en Frans Rosmalen en de studenten Ben Ackermans en Frans Thijssen.
Mijn volle aandacht kon ik aan dit proefschrift wijden in de wetenschap dat de bepalingen van T4, T3 en vrij T4 ten behoeve van de klinieken in goede handen waren bij Annelies Faber, Helga Wardenier, Rob Hermsen, Ferry Hübner en Jan Rijken.
Bij het literatuuronderzoek werd grotendeels gebruik gemaakt van de faciliteiten van de bibliotheek van het Praeklinische Instituut (hoofd: E.de Graaff).
De illustraties werden snel en goed verzorgd door de heren H.M.Berris, W.P.J.Maas en C.P.Nicolasen van de Medische Tekenkamer (hoofd: W.P.J. Maas) in samenwerking met de afdeling Medische Fotografie (hoofd: A.Th.A.I.Reynen).
Het manuscript werd persklaar gemaakt door Marlou Majoor.

Curriculum Vitae.

De schrijver van dit proefschrift werd op 30 september 1944 te Amsterdam geboren. In 1961 behaalde hij aldaar het diploma H.B.S.-b aan het toenmalige Christelijk Lyceum. In hetzelfde jaar begon hij zijn studie in de scheikunde aan de Vrije Universiteit te Amsterdam. Het doctoraal examen, met Analytische Chemie (Prof.dr. E.van Dalen en Prof.dr. K.W.Gerritsma) als hoofdvak en Biochemie (Prof.dr. R.J. Planta) als bijvak, werd afgelegd in 1970, waarna indiensttreding bij de afdeling Endocriene Ziekten (hoofd: Prof.dr. P.W.C.Kloppenborg) van de Kliniek voor Inwendige Ziekten (hoofd: Prof.dr. C.L.H. Majoor) van het St.Radboudziekenhuis te Nijmegen. Sindsdien heeft hij zich beziggehouden met de ontwikkeling van schildklierhormoonbepalingen en de analytisch-chemische aspecten van hormoon-eiwitinteracties.

STELLINGEN

bij het proefschrift

Symmetric and equilibrium dialysis for the measurement of free iodothyronine and steroid hormones in blood

H. A. ROSS

1

Bij evenwichtsdialyse van serum krijgt de concentratie van vrij, niet aan eiwit gebonden hormoon dezelfde waarde als verkregen wordt bij verdunning van het serum tot een volume gelijk aan de som van de volumina van dialysaat en dialysand

(dit proefschrift)

2

Maatregelen gericht tegen - door osmotische druk veroorzaakte - verandering van de verhouding tussen de volumina van dialysaat en dialysand tijdens evenwichtsdialyse, moeten in het algemeen als overbodig worden beschouwd

(dit proefschrift)

3

De interpretatie van wiskundige modellen voor ligand-bindingsevenwichten wordt aanzienlijk vereenvoudigd wanneer dissociatie- in plaats van associatie-constanten worden gebruikt

(dit proefschrift)

4

De term „aspecifieke binding" wordt zowel gebruikt voor ligandbinding met lage affiniteit als om het effect van onvolkomenheden in de scheiding van vrij en gebonden ligand aan te geven Deze dubbelzinnige term zou daarom in het spraakgebruik vervangen moeten worden door b v „lage affiniteitsbinding" waar het de eerste en „schijnbare binding" waar het de tweede betekenis betreft

5

Voor het onderzoek naar systematische verschillen tussen twee bepalingsmethoden kan het toetsen van het quotient van de uitkomsten van gepaarde metingen zinvoller zijn dan toepassing van lineaire regressie analyse

6

Voor het opzetten van een kwaliteitscontrole voor steroidreceptorbepalingen dient allereerst te worden gelet op de kwaliteit van de te volgen berekeningsmethode

litt Braunsberg H Hammond K D Methods of steroid receptor calculation An interlaboratory study J Steroid Biochem **11**, 1561 (1979)

7

De door Vigersky et al beschreven methode ter bepaling van de dissociatie-snelheden van de complexen van testosteron en oestradiol met testosteron-oestradiol bindend globuline is voor dit doel niet geschikt.

litt Vigersky, R A, Kono, S, Sauer, M, Lipsett, M B, Loriaux, D L.· Relative binding of Testosterone and Estradiol to Testosterone-Estradiol Binding Globulin J Clin Endocrinol Metab **49,** 899 (1979)

8

Het quotient van de serum concentraties van thyroxine (T4) en thyroxine-bindend globuline (T B.G.) is op theoretische gronden geen goede indicator voor de spiegel van het vrije T4.

9

De door Tamagna et al. gevonden aanwijzing voor een verhoogde spiegel van totaal en vrij thyroxine in het bloed van een patient met het hyperviscositeits-syndroom t.g.v. Waldenström's macroglobulinaemie kan uitgelegd worden als een noodzakelijk gevolg van de verhoogde serumviscositeit en behoeft niet te berusten, zoals de auteurs menen, op een artefact bij de bepaling van T4.

litt · Tamagna, E, Hershman, J, Premachandra, B. N. Circulating thyroid hormones in a patient with hyperviscosity syndrome Clin Chim Acta **93,** 263 (1979)
Dit proefschrift, ch II 5, eq (II-36) Bij constante flux (J) en vrije T4 fractie (f) moet daling van diffusiecoefficient DI door verhoogde viscositeit leiden tot een verhoogde waarde van totaal T4 (c) en vrij T4 (fc)

10

De stad Nijmegen is nog steeds een endemisch strumagebied.

litt: Kelly, F C, Snedden, W W in Endemic Goitre, W H O Geneva 1960
Pasma, F Verbreiding, oorzaken en bestrijding van struma in De endemische krop in Nederland, G O -T N.O Assen 1959
Beex, L V. A M Waarneming (niet gepubliceerd) gedaan tijdens het bevolkingsonderzoek naar borstkanker te Nijmegen, 1975-1977
Bij 500 steekproefsgewijs onderzochte vrouwen met leeftijd tussen 35 en 65 jaar bleek de strumafrequentie ongeveer 25% te bedragen

11

De bewering van Goodenow en Kaplan, dat RNA-afhankelijke DNA-polymerases van diverse type C RNA-tumorvirussen structureel sterk verwant zijn, wordt onvoldoende door hun experimenten ondersteund

litt : Goodenow, R S , Kaplan, H S Characterization of the reverse transcriptase of a type C RNA virus produced by a human lymphoma cell line Proc Natl Acad Sci (USA) **76,** 4971 (1979)

12

Wanneer men de kwaliteit van mens- en sociaalwetenschappelijk onderzoek wil beoordelen aan de hand van maatstaven, ontleend aan de natuurwetenschappen, dient men zich te realiseren dat de onderzoeksobjecten - mensen en groepen van mensen - zich niet uitermate lenen voor in vitro bestudering.

litt : Hoogerwerf, A Bèta of Gamma? Intermediair **16,** (5), 1 (1980)

13

Het Christen-Democratisch Appèl behoeft voor een naamsverandering in Centrum-Democratisch Appèl noch haar initialen, noch haar politiek te wijzigen